MEMBRANE PROTEIN FROM THE LYMPHOCYTE

ABSTRACT

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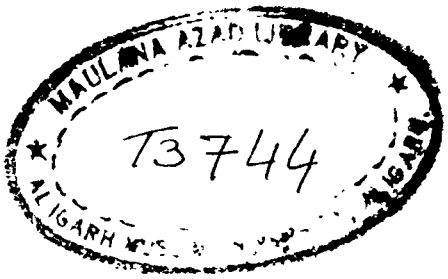
A thesis Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in Biochemistry in the Faculty of Medicine of the Aligarh Muslim University
ALIGARH
1989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ABSTRACT

One of the important lymphocyte membrane protein is immunoglobulin receptor which specifically recognizes the Fc portion of the IgG molecule. The membrane receptor has been identified on variety of cells including lymphocytes, monocytes, polymorphonuclear cells and are implicated in functions such as antibody dependent cell mediated cytotoxicity, suppression of antibody synthesis in B cells and phagocytosis in macrophages.

Although IgG receptors obtained from different sources have been found to show species dependent differences in pH stability and structure, studies on receptors from species other than human, murine and guinea pig are yet to be carried out. Further, despite the known functional significance of self association of the receptor, its aggregating tendency in aqueous buffer devoid of detergent remains to be systematically investigated. With this aim in view, we isolated and purified receptor from hitherto uninvestigated source i.e. goat peripheral blood lymphocytes. After its purification and characterization its tendency to undergo aggregation in aqueous medium was studied at different pH and ionic strength.

The presence of IgG binding protein on goat peripheral blood lymphocyte was detected by the specific

binding of FITC conjugated aggregated IgG to lymphocytes in 10 mM sodium phosphate buffer pH 7.4 containing 0.15 M NaCl, 1% BSA and 0.2% NaN_3. The binding of FITC conjugated aggregated IgG to goat peripheral blood lymphocytes increased with the increase in the concentration of aggregated IgG from 2 to 9 μg. Saturation in binding sites on 4.5×10^7 cells could be achieved with 0.7 μg/ml of aggregated IgG.

Binding of human IgG monomer, aggregated IgG and $F(ab_2')$ fragment to peripheral goat blood lymphocytes was studied by ELISA where the IgG or its derivatives bound to the lymphocyte surface receptors were assayed by using peroxidase conjugated $F(ab_2')$ of antihuman IgG under different conditions of pH and ionic strength. The binding of IgG and its derivatives was studied at different pH values in the pH range 3-8 which was maintained either by 0.06 M sodium acetate (pH 3-5) or by 0.06 M sodium phosphate (pH 6-8). It was found that maximum binding occurred with heat aggregated IgG followed by IgG monomer and its fragments $F(ab_2')$.

Furthermore the pH dependence of aggregated IgG was found to be more pronounced than the binding of IgG monomer to cells in the entire pH range (pH 3-8). Maximum binding of aggregated IgG took place at pH 6.0 below or above which the change in pH caused considerable

reduction in binding. The binding of monomeric IgG decreased gradually and monotonically as the pH decreased from pH 8 to pH 3. A small but experimentally significant binding of $F(ab_2')$ to cells was observed, it was completely abolished below pH 4.0.

The effect of ionic strength on binding of IgG and its derivatives to goat peripheral blood lymphocytes was studied in 10 mM phosphate buffer pH 7.6 at 37°C. Increasing concentration of NaCl upto 0.8 M caused significant decrease in the binding. The ionic strength dependence of the binding was more pronounced with heat aggregated IgG.

Goat peripheral blood lymphocyte receptor for IgG was isolated by affinity chromatography both from cell homogenate as well as from isolated membrane. The lymphocyte membrane was prepared by hypotonic lysis of cells by freezing in 10 mM tris HCl buffer pH 7.0 containing 1 mM $MgCl_2$ and 1 mM KCl followed by centrifugation. The membrane fraction was identified by the measurement of 5'- nucleotidase activity against AMP in 55 mM Tris HCl buffer pH 8.5 containing 5.5 mM magnesium chloride. The membrane fraction showing a 5' - nucleotidase activity of 1.27 µg Pi/mg/min which was expectedly higher than 0.33 µg Pi/mg/min measured for cell homogenate was used in the isolation of the receptor.

The membrane or the whole cell were solubilized in 10 mM sodium phosphate buffer pH 7.4 containing 0.15 M NaCl, 0.5.% NP-40, 2 mM PMSF,3 mM EDTA and 10 mM iodoacetamide and after treating the mixture with BSA Sepharose the protein was incubated with heat aggregated IgG Sepharose 4B affinity column. The protein yield of the affinity purified receptor was less than 1% . The receptor preparations, both from the isolated membrane and whole cells were identical in (a) reactivity towards IgG aggregated and Con A Sepharose gel (b) gel filtration behaviour (c) subunit molecular weight and (d) finally in their tendencies to undergo pH and temperature dependent aggregation.

The receptor preparation was homogeneous with respect to size as indicated by SDS-PAGE where it moved as a single protein band with a subunit molecular weight of 14 kDa both in the presence and absence of 0.2 M mercaptoethanol. These results suggest that the subunits in the receptor are held together only by noncovalent forces.

The IgG binding receptor was found to be a glycoprotein devoid of sialic acid residues. The hexose content of the receptor as determined by phenol sulphuric acid method was found to be 11% (w/w) i.e. about 9 moles of hexose per 14000 g of the receptor. As the receptor

specifically interacted with Con A-Sepharose column its carbohydrate moieties must include specific sugar residues such as mannose/glucose. In 10 mM sodium phosphate buffer plus 0.15 M NaCl and 0.1% deoxycholate the IgG receptor absorbed maximally at 278 nm. The measured fluorescence excitation and emission spectra of the receptor in the same buffer showed excitation and emission maxima near 277.6 nm and 341.4 nm, respectively. These results suggest that the aromatic amino acid residues of the receptor must include tryptophan residue(s).

On gel filtration of the IgG binding receptor at 25°C on an HPLC Shim Pack Diol 150 column equilibrated in 10 mM sodium phosphate buffer pH 7.4 containing 0.5 M NaCl, 60% of the receptor eluted with a retention time of 6.46 min which yielded a molecular weight of 67000 and Stokes radius of 3.48 nm. Other hydrodynamic parameters that were calculated from the Stokes radius included diffusion coefficient (5.2×10^{-7} cm^2/sec) and frictional ratio (1.3). These data are consistent with a nonglobular conformation of the major fraction of the receptor in aqueous buffer solution devoid of detergent. The minor fractions included higher aggregates with molecular weights of 119 kDa and 94 kDa.

The IgG binding receptor showed pH and temperature dependent association. In 0.5 M acetic acid pH 2.8, and at

37°C the receptor was found to exist primarily as 31 kDa species. On lowering temperature,at the same pH, to 4°C association of 31 kDa species into 67 kDa species occurred. The trimer and tetrameric forms of 31 kDa species which were absent in 0.5 m acetic acid pH 2.8 at 37°C appeared in 10 mM sodium phosphate, 0.5 NaCl pH 7.4 at 25°C.

Thus both acidic pH as well as increase in temperature favoured dissociation of IgG binding protein to 31 kDa from. As revealed by SDS-PAGE results the 31 kDa species itself seems to be a dimer of 14 kDa subunits held together by noncovalent interactions. Since increase in temperature favours dissociation of the goat IgG binding receptor the self association of the goat lymphocyte receptor is likely to be exothermic in nature.

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A thesis Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in Biochemistry in the Faculty of Medicine of the Aligarh Muslim University
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1989

CERTIFICATE

I certify that the work presented in the following pages has been carried out by Mr.Krishnan Hajela and that it is suitable for the award of Ph.D. degree in Biochemistry of the Aligarh Muslim University, Aligarh.

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ACKNOWLEDGEMENTS

I would like to express my deep sense of gratitude to Professor A. Salahuddin for his keen interest and valuable guidance in the completion of the thesis right from the very beginning. It is impossible to evaluate the moral encouragement he always provided whenever I felt myself insufficient to carry on the project.

I would like to gratefully acknowledge kinc help provided by Professor Intisar Hussain in the initial stages of my work.

I am grateful to Professor Rashid Ali, Head Of the Department of Biochemistry, for providing all the necessary facilities.

I sincerely acknowledge the cooperation extended to me by my seniors, Dr. Sibghatullah, Dr. Naheed Bano, Dr.Sudhir Kumar Agarwal, Dr.Rasheedunisa Begum, Dr.Tauseef Saeed Khan, Dr. Saad Tayyab, Mr. Nizamuddin Ahmad and Mrs.Khushtar Salman.

My warmest thanks are due to Mrs. Nausheen Wasem, Mrs. Renu Tyagi, Miss Najma Ali, Miss Praveen Salahuddin, Mrs. Manjeet Kaur, Mr. Khalid Fazili, Mr. Mir Muzzafar, Mrs.Sadhana Sharma and Miss Seema Hasan for the help provided by them.

I also extend my thanks to Miss. Sabiha, Mrs. Zia Afroz, Miss. Azra Abid, Mrs. Zoya Galzie and Miss. Haleema Zeba whose help were always at my disposal.

Appreciation is also due to the members of the non teaching staff, particularly to Mr. Amir Ali and Mr. Lal Mohammad Khan for their help and cooperation.

Thanks are also due to my sister Namita whose love and cooperation helped me all these years.

Financial assistance in the form of Senior Research Fellowship from Council of Scientific and Industrial Research is gratefully acknowledged.

Krishnan Hajela

Aligarh (Krishnan Hajela)

May, 1989

DEDICATED TO MY AUNTS

Prof. Miss Pushpa Hajela

and

Late Mrs. Pushplata Johri

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LIST OF ABBREVIATIONS

ADCC	Antibody dependent cell mediated cytotoxicity
Agg	Aggregated
Agg-FITC-IgG	aggregated IgG conjugated with FITC
AMP	Adenosine monophosphate
cAMP	Cyclic adenosine monophosphate
APC	Antigen presenting cells
CD	Cluster of differentiation
CH	Constant region of heavy chain
$C_\gamma 1$	First domain in the constant region of IgG
$C_\gamma 2$	Second domain in the constant region of IgG
$C_\gamma 3$	Third domain in the constant region of IgG
CON A	Concanavalin A
DEAE	Diethyl Amino Ethyl Cellulose
EAC	Erythrocytes Antibody Coated
Fab	Fragment antigen binding
$F(ab_2')$	Fragment antigen binding divalent
FACS	Fluorescence Activated Cell Sorter
FITC	Fluorescein Isothiocyanate
Fc	Fragment crystallizable
FcR	Receptor for Fc region of immunoglobulin
$Fc_\gamma R$	Receptor for Fc region of IgG
$Fc_{\gamma 2a}R$	Receptor for Fc region of IgG 2a
$Fc_{\gamma 2b}R$	Receptor for Fc region of IgG 2b

FcR	Receptor for Fc region of IgA
$Fc_{\delta}R$	Receptor for Fc region of IgD
$Fc_{\varepsilon}R$	Receptor for Fc region of IgE
$Fc_{\mu}R$	Receptor for Fc region of IgM
gp	Glycoprotein
GPBL	Goat Peripheral Blood Lymphocytes
HAIgG	Heat aggregated IgG
HLA	Human Leukocyte Antigens
HPLC	High Performance Liquid Chromatography
Ig	Immunoglobulin
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgG_1	Immunoglobulin subclass in human
IgG_2	Immunoglobulin subclass in human
IgG2a	Immunoglobulin subclass in mouse
IgG 2b	Immunoglobulin subclass in mouse
IgM	Immunoglobulin M
IgG BF	Immunoglobulin G binding Factor
IL_2	Interleukin-2
Lyb	B lymphocyte surface antigen
MHC	Major histocompatability antigen
NP-40	Noniodet P-40
NK	Natural Killer Cell
OPD	Orthophenylene diamine

PBS	Phosphate Buffered Saline
pFc'	Pepsin fragment of Fc
PLA_2	Phospholipase A_2
PMSF	Phenyl methyl sulfonyl fluoride
RBC	Red Blood Cells
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
sFcR	Soluble Fc Receptor
SRBC	Sheep Red Blood Cells
SRS-A	Slow Reacting Substance of Anaphylaxis
$T_{C/cyt}$	T cytotoxic cells
T_H	T helper cell
T_S	T suppressor cell
TRF	T cell replacing factor
VH	Variable region of heavy chain
WBC	White Blood Cells.

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I. INTRODUCTION

According to the fluid mosaic model the lipid bilayer constitute the basic structure of a membrane .The amphipathic protein molecules are partially or wholly integrated into the lipid bilayer (Singer and Nicolson, 1972). The cell membrane represents the dynamic, fluid structure in which both the lipid and protein molecules are free to move about in the plane of membrane (Singer and Nicolson, 1972). The membrane protein molecules mediate various functions which include (a) transport of specific molecules in or out of the cell (b) catalysis of the membrane associated reactions, and (c) transduction of receptor mediated chemical signals from the extracellular environment to the cell interior.

Lymphocyte membrane is directly involved in the immune response. The membrane associated receptor interacts specifically with the antigen prior to antigen stimulated proliferation of immunocompetent cells (Roitt, 1988). Initially the ligand interacts with specific antigen receptor on B/T cells. This specific ligand may be an antigen (or its processed form), surface receptor of an accessory cell and/or a mitogen. The signal generated by this specific interaction is transmitted into the cell interior by a mechanism which is still poorly understood. However the consequences of such interactions of the

antigen with the lymphocyte is the growth of lymphocytes followed by its division and differentiation into immunocompetent memory cells and effector cells (Roitt, 1988). Interaction of antigen with cell surface of B cell transforms a resting B cell into a plasma cell which is capable of secreting antibodies . It should be pointed out that the proliferation of B cells is also regulated by T cells and other cells including macrophages. In cell mediated immune response, interaction of antigen with receptor on T cells can activate helper, suppressor or cytotoxic T cells. The MHC antigens are essential for reaction of immune recognition (Roitt et al., 1985). Different MHC antigens are recognized by different T cell types. These interactions are summarized in Figure I.

The cytotoxic T cells recognize foreign antigens on the target cell. Here the foreign antigen is a virus which in mice associates with H_2K and H_2D antigens on the target cells. In human these MHC antigens are replaced by HLA A and HLA B antigens. B cells or other antigen presenting cells also interact with the antigen which are processed and presented in association with MHC class II molecules (H-2I in the case of mice) to specific T_H cells which can cooperate with B cells for the production of antibodies. T_H cells also produce lymphokines which help macrophages to kill intracellular microorganisms.

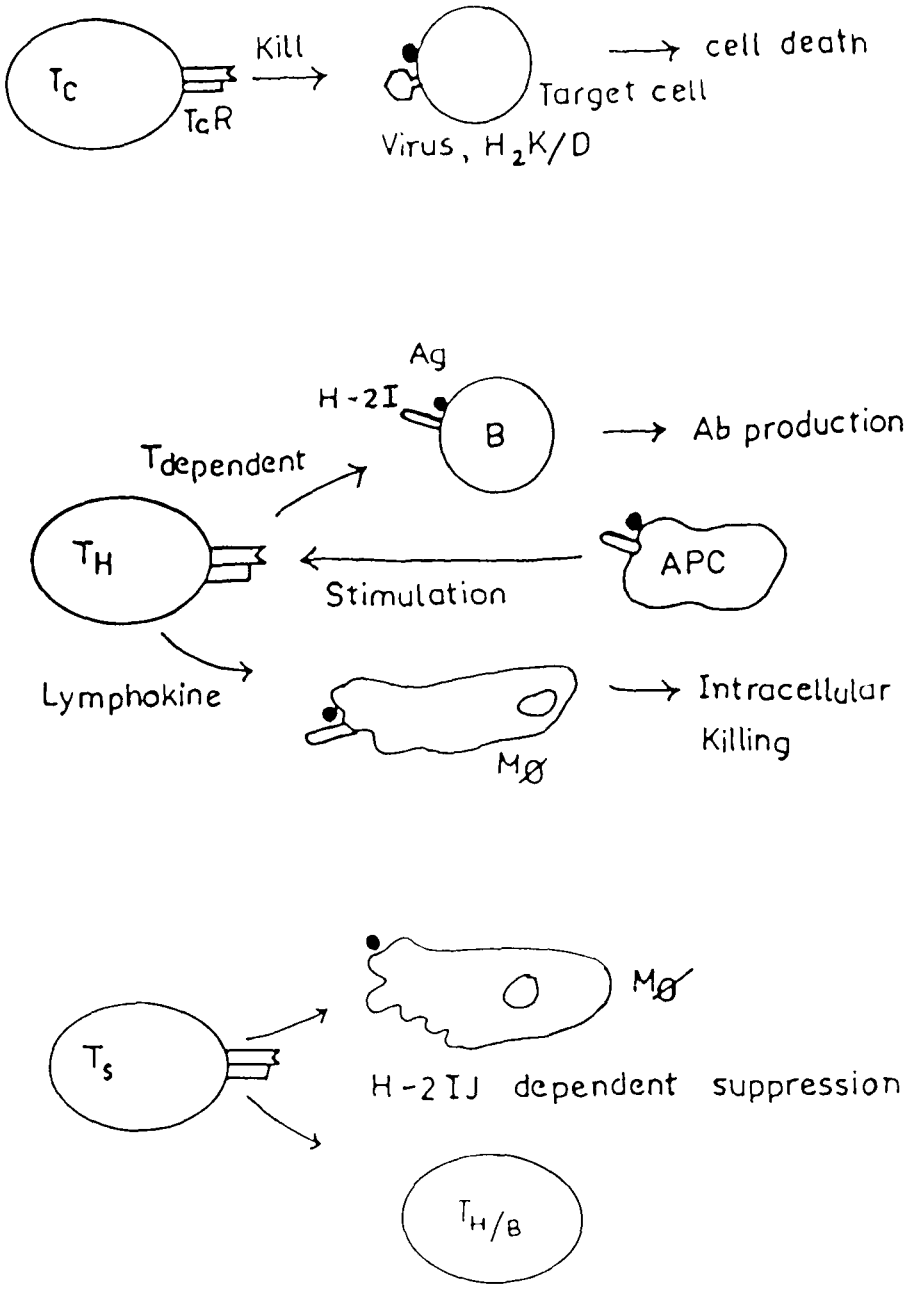


Figure 1

Summary of MHC restriction in immune cell interaction. (a) cytotoxic T cells ,H_2 K\D MHC class I restricted (b) Helper T cells H2-I MHC II restricted (c)Suppressor T cells H2I-J MHC class II restricted .See text for details.

Suppressor T (T_S) cells specifically suppress the action of macrophages, B cells and T_H cells which are induced by foreign antigen. The interaction between T_S cells and their target is most efficient when they both share the same I-J (MHC II) haplotype.

A large number of specific protein antigens on lymphocyte surface have been identified and some times partially characterized (Williams and Barclay, 1986, Horejsi and Bazil, 1988). Some of the important ones are listed in Table I. Most of the receptors appear to be glycoprotein in nature. The association of these receptors with other membrane proteins and elements of cytoskeleton as well as self association of receptors have considerable functional implications. A schematic diagram of lymphocyte membrane showing major glycoprotein antigens is given in Figure 2. The functional association of lymphocyte surface glycoproteins are given in Table II.

Cell Surface Receptors:

A receptor is defined as a membrane associated molecule specific for a given ligand which upon interaction with its ligand takes part in a precise function. The binding should be saturable, reversible and should have high affinity. The receptors recognised in lymphocytes membrane can be divided into four different

TABLE I

Glycoprotein or protein antigens identified on lymphocyte surface[a]

Antigen	Glycoprotein (gp) and protein chains Mr x 10^{-3}	Tissue Distribution
IgM+other classes	2xgp 78(M):2xp23(L)	Ag receptors on B Cells
TcR	gp 40-43:gp 44-49	T lymphocytes Ag receptor
MHC Class 11	gp 33(α):gp 28(β)	B, dendritic, macrophages epithelial cells
MHC Class 1	gp 45: gp 12 (β_2 m)	Wide,but not on neuronal glial cells
TL	gp 44: p12 (β_2 m)	Leukemias,thymocytes
CD1		Langerhans, possible homology of TL
L-CA	gp 180-240	Leukocytes only
LSGA	gp 95	Thymocytes,polymorphs,stem cells, plasma cells, brain
Thy 1	gp 18.7	Neuronal, fibroblast,connective tissue, haemopoetic cells
Pan T (CD2)	50	Thymocytes, T
Pan T (CD3)	gp 19-29	Thymocytes, T
CD5		Lymphocytes, leukemic
CD6	120	T.B. subsets
CD7	41	Thymocytes, T
T helper [CD4]	gp 55	Thymocytes, T helper, macrophages
T cytotoxic [CD8]	gp 20-35 gp 32-43	Thymocytes, T cyto/supp. Some rat,human, NK cells
LYb-2	45	B cells
LYb-3	68	B cells subset
LYb-8	gp 95-105	B cells subset

a. Williams and Barclay, 1986.

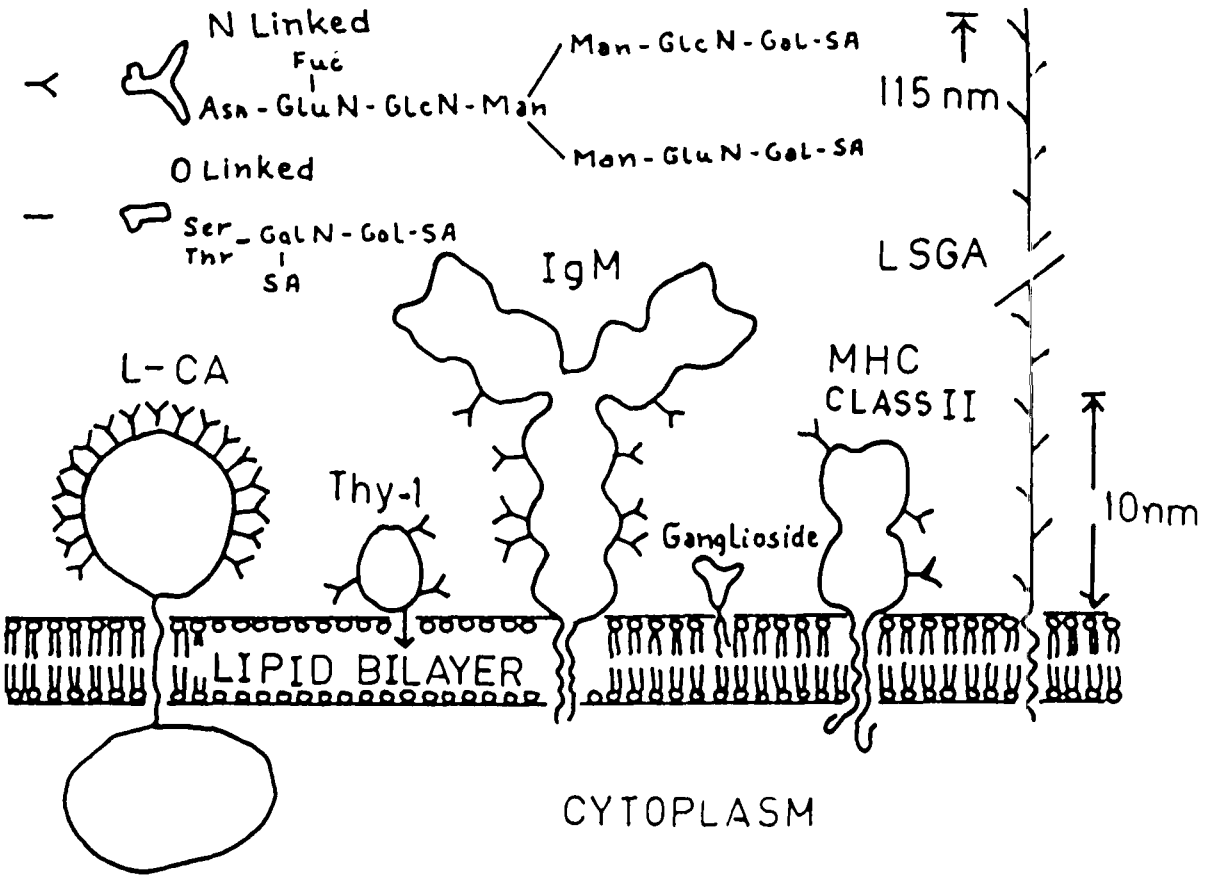


Figure 2

Some abundant lymphocyte membrane glycoproteins(drawn to scale)present on the cell surface. The carbohydrates could not be drawn to scale on the model but are shown to the scale of their greatest cross sectional area in the upper left hand part of the figure. (Willams and Barclay, 1986).

TABLE II

Lymphocyte surface glycoprotein with known functions or functional associations[a]

Molecules with known functions

1. Surface Ig : acts as antigen receptor on B lymphocytes.

2. T lymphocyte antigen receptors: monoclonal antibodies recognize idiotypes of T cell clones and inhibit functions.

3. Class I MHC : restriction of antigen presentation, mainly to cytotoxic T cells.

4. Class II MHC: restriction of antigen presentation mairly to helper T cells.

5. IL-2 receptor binds to IL2 growth factor, interaction essential for division of activated T cells.

6. Transferrin receptor present on many dividing and other cells, binding of transferrin can be essential for cell growth.

7. Insulin receptor:mediate effect of insulin on variety of cell types.

Molecules with functional associations

1. Fc receptors*: binds complexes of Ig, implicated with various effector functions.

2. T helper: W3125 antibody inhibits rat T cell division and IL2 production, anti-human T4 blocks cytotoxicity by $T4^+$ killer cells. Mouse L3T4 inhibits T cell activation.

3. T cyto/supp : Some antibodies inhibit T cell cytotoxicity, antibody to rat molecules inhibit generation of Tcyt cells.

4. LYb-2 antibodies inhibit generation of Ab secreting cells, is a B cell mitogen

---

a. Williams and Barclay, 1986.

* Fc receptor, FcR, Ig receptor, Ig binding protein Ig binding receptor have been used synonomously in this thesis.

categories (Marchalonis and Galbraith, 1987). These are

(a) Antigen Specific Receptors

(b) Triggering Receptors

(c) Supportive Receptors

(d) Miscellaneous Receptors

(a) Antigen Specific Receptors

The first category includes receptors involved in antigen binding. For example, the membrane immunoglobulins on B cells, antigen binding T cell receptor and MHC restricted T cell receptor. The antigen binding site of membrane immunoglobulins is formed by gene rearrangement involving V, J and C region segments. (Roitt, 1985). The antigen specific receptor of T lymphocytes are structurally related to immunoglobulin. (Horejsi and Bazil, 1988).

Two different types of human T cell receptors have been recognised.

The receptors called Ti is a disulfide linked dimer comprising of α and β chains and is expressed in most of the T cells. The other T cell receptor contain the polypeptide chains $\gamma\delta$ and are expressed on embryonic thymocytes and on small population of T cells having a natural killer activity. Both the receptors are non

covalently associated with CD3 complex. The dimeric cell surface receptor recognizes antigen while CD3 complex is probably involved in the transmission of the external signal into the cell's interior.

(b) Triggering Receptors

These receptors have a specific function of determining cellular responses by driving lymphocytes along more or less defined pathways. Thus they are termed as "triggering" or "determining" receptors. This category includes receptor for B cell growth factors (BCGF), B cell differentiation factor (BCDF), interleukin receptor (IL2) or T cells and mitogen receptor. The proliferation of B cells is regulated by the binding of BCGF derived from T cell and BCDF. Similarly binding of IL2 to its receptors on T cell lead them into a proliferative phase.

(c) Supportive Receptors

This category includes receptors for hormones like insulin, growth hormones, steroids, and also for carrier proteins like transferrin, low density lipoproteins, transcobalamines etc.

(d) Miscellaneous Receptors

Among this group of receptors are included receptors for adrenalin, histamine, acetylcholine, interferon,

third component of complement and Fc receptors. The Fc receptors were detected on macrophages as early as 1966 (Berken and Benacerraf, 1966) and on T and B cells in 1972. (Lee and Paraskevas, 1972). Paraskevas et al. (1972) used the term Fc receptor for the cell surface molecules recognizing Fc region of IgG. The structural segment recognizing Fc receptor is being described below:

The structure of Immunoglobulins

The structural features of immunoglobulin as studied by Putnam (1987) are schematically represented in Figure 3. The immunoglobulin consists of two identical heavy chains and two identical light chains which are linked by interchain disulfide bonds and noncovalent interactions. The heavy chain consist of 4 domains of approximately 110 amino acid residues each with regularly spaced intrachain disulfide bonds. The variable region is made up of one domain V_H while the constant region is made up of three domains C_H1, C_H2, C_H3. The light chain contains one variable and one constant region. The Fab region, comprising of V_H and V_L domains, directly interacts with the antigen. The domains C_H and C_L provide structural support to antigen binding site. (Klein et al., 1979). The Fc region of IgG comprises of C_H2 and C_H3 regions and offers site for binding of various molecules to IgG. These molecules include C1q, C1s, C4 and Fc

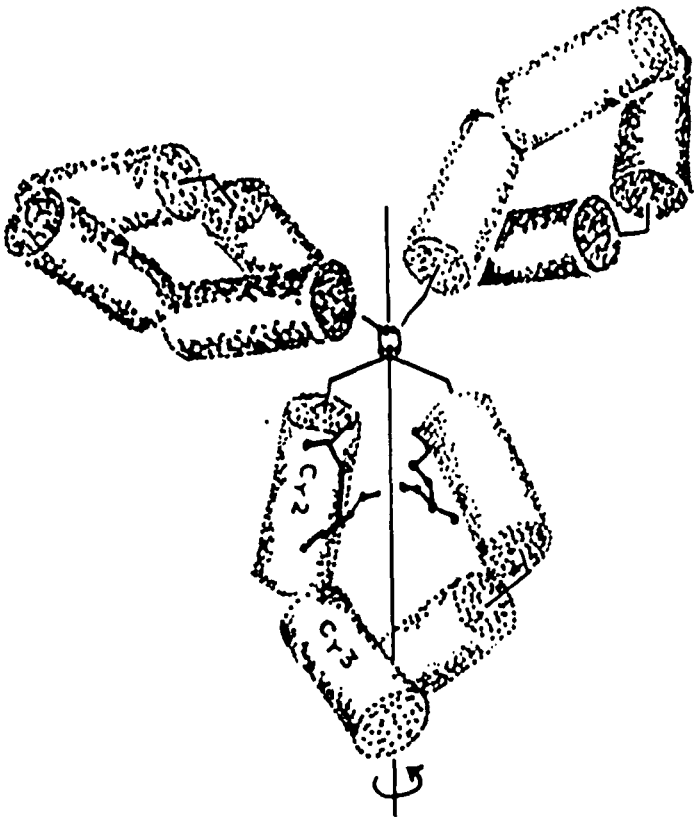


Figure 3

A schematic model of IgG_1 molecule based on the X ray crystallographic data. Each cylindrical domain corresponds to a homology region apparent in the amino acid sequence of the heavy and light chain. Note the absence of non-covalent interaction between $C\gamma 2$ domains which are separated by a solvent filled space. The lined filled circles represent the carbohydrate prosthetic group covalently attached to each $C\gamma 2$ region. The molecular two fold axis is shown (Deisenhofer and Huber, 1983).

receptors (Burton, 1985). The internal sequence homology between domains suggest a common three dimensional structure in all the V and C domains. As can be seen in Figure.4 each domain forms a globular structure and is made up of seven polypeptide stretches forming β pleated structures (Burton 1985). The four strands FX_1, FX_2, FX_3 and FX_4 on one hand and strands FY_1, FY_2, and FY_3 on the other form a sandwich like structure as shown in Figure 4. There are some notable differences in the V and C domains (Deisenhofer and Huber, 1983). Domain C lack 2 strands. Further, the length of the loop in C domain is different from the length of the loop in the V domain. Domain-domain interactions are stabilized by noncovalent interactions. The C_H3 domains are associated tightly through lateral noncovalent interactions whereas the C_H2 domains are separated by solvent filled space (Deisenhofer and Huber, 1983). Carbohydrates are located in the inner surface of C_H2 domain. Its functional role is yet to be explained fully. The C_H3 domain of human IgG contains four extensive solvent accessible hydrophobic patches (Burton, 1985), two of which interacts with protein A. The C_H2 domain also contains four similar patches but only one forms the protein A binding site at C_H2-C_H3 interface. Fc receptor also interacts with the C_H2/C_H3 domain of the Fc region of immunoglobulin molecules.

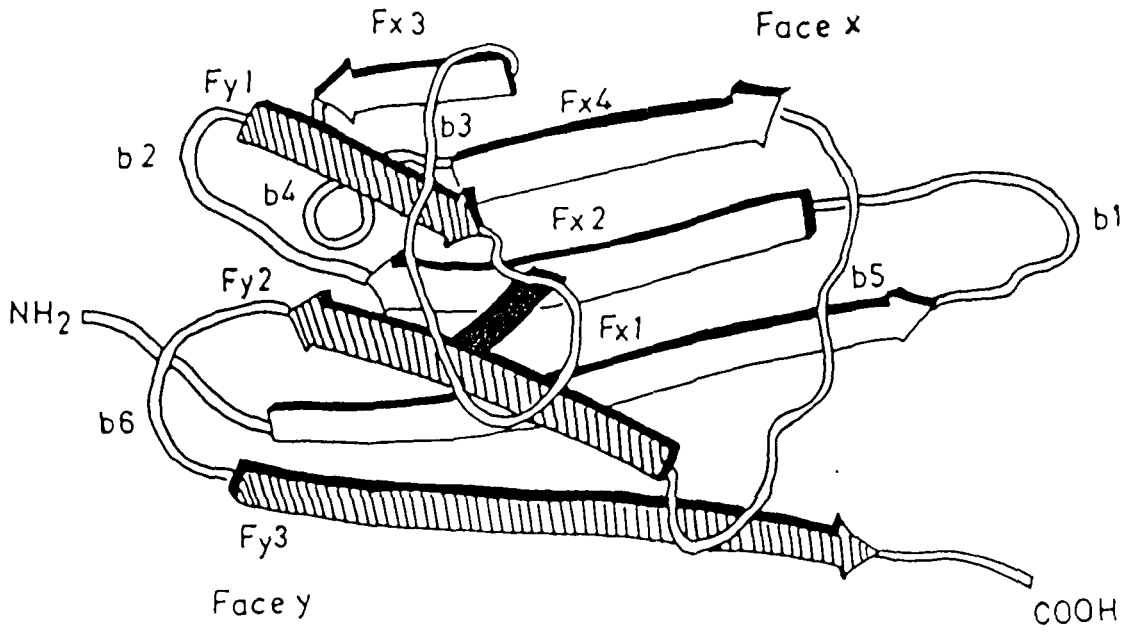


Figure 4

The three dimensional structure of a immunoglobulin domain. The segements F x 1-4 (unshaded) and Fy 1-3 (shaded) form two parallel faces of antiparallel pleated sheets linked by intra chain disulfide link (filled rectangle). Between pleated segements are other segements (b 1-6) forming helices, bands and other structures (Burton, 1985).

Occurrence of Ig receptor

Ig receptor are expressed in all cell types particularly in the immune process. In this thesis we have focussed attention only on the receptor for heat aggregated IgG. Receptors for different classes of immunoglobulins have been recognized on immunologically important cells. These are listed Table III.

As can be seen, surface receptor for IgG (Fc R) have been found on a variety of cells including lymphocytes, monocytes and polymorphonuclear leukocytes (Lydyard and Fanger, 1982) whereas the expression of receptor for the other classes of immunoglobulins is more restricted. Thus IgM receptors ($Fc_{\mu}R$) are found only on lymphoid cells (Lydyard and Fanger, 1982), while those for IgA ($Fc_{\alpha}A$) are present on lymphoid cells as well as on monocytes and granulocytes (Fanger et al, 1980).

IgE ($Fc_{\epsilon}R$) receptors are found on mast cells, basophils, monocytes and some subpopulations of lymphocytes. (Melewicz and Spiegelberg, 1980). Lymphocytes also contain receptor for IgD i.e. $Fc_{\delta}R$ (Sjoberg, 1980). Two distinct $Fc_{\gamma}Rs$, one binding to IgG_2 and other binding to IgG_2 as well as IgG_1 were identified on guinea pig macrophages (Sugiyama et al., 1981). Rat basophilic leukemia cell line HL 3 contains two functionally

TABLE III

Expression of individual Ig binding receptor types on immunologically important cell types[a][b]

Cells	Ig binding receptor[c] for						
	IgG_1/IgG_2B	IgG_{2A}	IgG_3	IgM	IgA	IgE	IgD
T lymp	+	+	0	+	+	+	+
B lymp	+	+	0	+	+	+	+
Non T non B	+	+	0	+	+	0	+
Macrophages, monoytes	+	+	+	+	±	+	0
Dendrities cells	+	+	0	0	0	0	0
K cells	+	+	+	+	0	0	0
NK cells	+	+	0	+	0	0	0
Eosinophils[d]	+			0	-	+	0
Neutrophils[d]	+			0	+	-	0
Basophils[d]				+		0	0
Mast cells[d]	+			0	0	+	0
Thrombocytes[d]	+			-	-	-	0

(a) Fornusek and Vetvicka, 1984.
(b) + presence of Ig binding receptor proved
- presence of FcR not proved
± contradictory data, 0 FcR lacking
(c) Distribution of FcR for individual IgG subclasses performed according to the situation in mice
(d) individual FcγR subtypes were not examined.

different receptors. One binds to IgE only and the other binds to IgG and IgE as well (Segal et al., 1981). Ig binding receptor are found on many tumor infilterated cells such as lymphocytes, macrophages, polymorphonuclear leukocytes, and also on some per cent of tumor cells. (Svennevig and Anderson, 1982).

Except lymphoid cells, Ig binding receptor have been found on many non-lymphoid cells also. These include mouse hepatic cell membrane (Shinohara et al., 1981), kuffer cells of normal human liver, human liver cell membrane (Shizohara et al., 1981), human and mouse spermatozoa (Sethi and Brandis, 1980) and ciliary process of rabbit eye(Peress et al1982). Rabbit yolk sac cells (Tsay et al., 1980) and human placental cells (Mikulska et al., 1982) have been found to contain Ig binding receptor. In these cells the receptors are implicated in the transfer of immunoglobulin G from mother to the foetus. Epithelial cells from neonatal rat small intestine express Ig receptor (Simister and Rees, 1983, Hobbs et al,1987) which mediate the transport of IgG from the gut to the lumen. Ig binding receptor has also been recognised on cells in human central nervous system (Nyland and Nilsen, 1982). These include pia and arachanoid cells of the leptomeningis, the stroma cells of arachanoid granulations, advential cells in the perivascular spaces of nervous tissue, the villi of

choroid plexus and on pericytes of some of the brain capillaries. However they are not present on brain parenchyma (Nylund and Nilsen, 1982) . Ig binding receptors have been detected on sheep, rabbit, cow, guinea pig, dog and chicken erythrocytes (Manghi et al., 1987). Staphylococcus contains several proteins binding immunoglobulins through Fc portion. These include protein A and four different types of Ig receptors (Lammler et al, 1986). Ig binding receptors are expressed both on mouse fibroblasts as well as Hereps simplex virus after the infection of the cell by the virus (Fornusek and Vetvicka, 1984).

Detection of Ig Receptors

There are several methods available for the detection and quantification of Ig binding receptor. These are briefly described below:

(a) Rosette formation with antibody coated erythrocytes

In this assay the erythrocytes are first coated with sub-heamagglutinating doses of antibody raised against it. The antibody coated erythrocytes (EAC) are then mixed with cells possessing Ig binding receptor.

As shown in Figure.5 Ig binding receptor bearing cells interact with the Fc portion of immunoglobulin bound

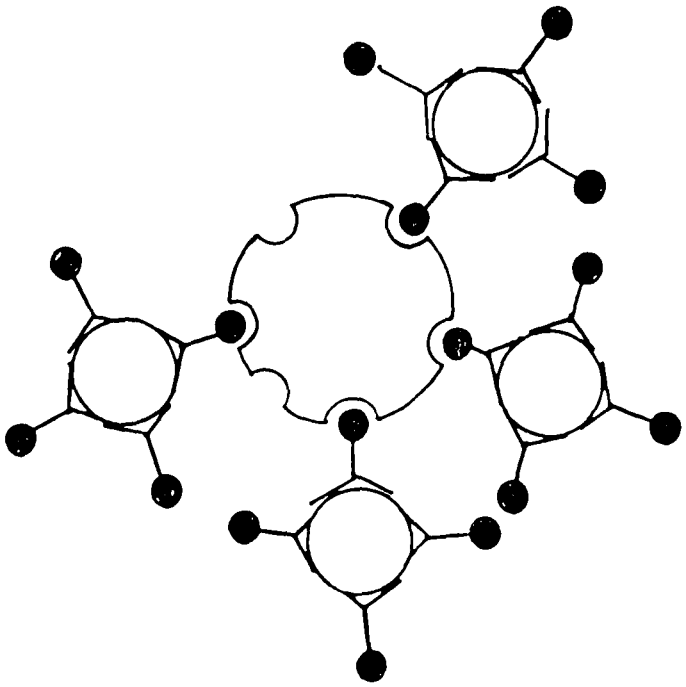


Figure 5

B cell rosette. A diagramatic representation of rosette formed with IgG (Y) coated erythrocytes binding to the receptor.

to the erythrocytes forming rosettes (Binns et al., 1983). The mixture is centrifuged and the pellet suspended in solution. The rosetted cells were counted under microscope. Generally sheep,ox or human erythrocytes are used. The sensitivity of the assay could be increased by using monoclonal antibodies against erythrocytes because larger amounts of antibodies could then be coated without agglutinating the cells (Diamond et al., 1978). IgG fractions can also be isolated from antisera obtained from hyperimmune animals. Presence of immunoglobulins of different isotypes may give erroneous results. Therefore erythrocytes coated with equivalent amount of $F(ab_2')$ fragment of antibody are used as control. Methods used to disaggregate tissues into single cells may damage Ig binding receptor expression. EAC rosetting assay has been used for the detection of Ig binding receptor on intact tissues (Kerbel and Elliot, 1983).

It should be pointed out that EAC rosette assay can only be used satisfactorily for the detection of Ig binding receptor on the cells. This method is inadequate for obtaining quantitative information for the binding of Ig binding receptor in terms of association constant etc.

(b) Detection of Ig receptor using molecular antigen-antibody complexes

In this assay antigen antibody complex is used in which antigen binding sites are occupied by antigen and Fc portion of the molecule is available for interaction with the free receptor or with the receptor bearing cells. In the immune complex either antibody or antigen or both can be labelled with fluorophore such as fluoroscein (or rhodamine) isothiocynate .The complex is incubated with the Ig binding receptor bearing cells, washed and analysed by fluorescent microscope. Cells expressing different Ig binding receptor can thus be separated by using Fluorescence Activated Cell Sorter. The sensitivity of the method can be substantially enhanced by using radiolabelled antibody or antigen.

(c) Detection of Ig binding receptor using aggregated or monomeric IgG

Aggregated immunoglobulins have also been used for the assay of Ig binding receptor. Immunoglobulins can be aggregated either by heating or crosslinking by chemical agents (Heusser et al., 1977) and the IgG aggregates of different sizes were separated by gel filtration on Sepharose 4B (Heusser et al., 1977) or by ultracentrifugation (Rasmussen et al., 1983). The

immunoglobulins can either be labeled with I^{125} or by fluorophore conjugation to enhance the sensitivity of the method.

We have used fluorometeric assay for detection of Ig receptor on goat peripheral blood lymphocytes. FITC was used for labelling the immunoglobulins prior to its aggregation in view of its higher quantum yield. If the complex of the aggregated IgG with receptor is to be monitored by fluorescence microscope, rhodamine isothiocyanate should be preferred. Radiolabelled monomeric imunoglobulins can also be used for this assay but for this binding to take place the affinity of Ig binding receptor for monomeric IgG should be high. From this method the association constants and the number of combining sites can be calculated using standard scatchard analysis (Unkeless and Eisen, 1975).

(d) Detection of Ig binding receptor using anti Ig binding receptor antibodies

Antibodies raised against Ig binding receptor have been used for the detection . Antibodies against Ig binding receptor of human IgM bearing lymphoblastoid tumor cells were raised in rabbit (Takacs, 1980). The anti Ig binding receptor serum reacted with all the cells from Ig binding receptor bearing surface IgM positive human

lymphoblastoid cell lines but not with Ig binding receptor negative human T cell lines. Several monoclonal antibodies have also been raised against Ig binding receptor (Shimamura et al, 1986, Mellman and Unkeless, 1980) which are used for its detection on various cells.

(e) Use of radioimmunoassay for detection of Ig binding receptor

In this method Ig binding receptor bearing cells are incubated with aggregated IgG and the decrease in IgG concentration is detected by measuring the free IgG concentration by radioimmunoassay (Frade et al., 1983).

(f) Detection of Ig receptor activity on nitrocellulose membrane

In this method, first of all Ig binding receptor or receptor bearing cells are immobilized on nitrocellulose membrane. Immunoglobulin solutions are then added and the immunoglobulin bound to nitrocellulose membrane via receptor is determined by an anti immunoglobulin antibody linked to peroxidase (Lammler et al., 1986). By this method it was possible to detect Ig binding receptor on different strains of staphylococci and streptococci. This method besides being semi-quantitative can be used to screen bacterial cultures of a large scale.

(g) Detection of Ig receptor activity using an enzyme linked immunosorbent assay

This is a sensitive method for assay of Ig binding receptor. The cells containing Ig binding receptor were first fixed to the wells of ELISA plates with glutaraldehyde. Subsequently purified immunoglobulins are added. The amount of bound immunoglobulins is detected by measuring the activity of an appropriate enzyme coupled with anti immunoglobulin antibodies . This assay has been used for the detection of isotype specific Fc receptors on T cell hybridomas (Kurita et al., 1985). In this study this assay has been used to detect goat peripheral blood lymphocyte Ig binding receptor.

Methods for isolation and purification of Ig binding receptor

Ig binding receptors have been generally isolated by affinity chromatography using immobilized ligands as the matrix. Alternatively Ig binding receptor complexes can be specifically precipitated with anti IgG. The specific precipitation of the receptor with antireceptor antibody can also be used in its isolation. One of the main problems in isolating the receptor is the availability of limited amount of receptor as compared to total membrane proteins. Further the cytophilic IgG has to be removed. This intrinsic immunoglobulin can be removed

by using protein A Sepharose columns. The columns can be monitored by measuring radioactivity after radiolabelling of the proteins. Since Ig receptor is a membrane protein it will be appropriate to isolate the membrane from the cell. The procedure usually used for the isolation of membrane from lymphocytes is as under. The cell membrane is disrupted either by nitrogen cavitation, sonication or hypotonic lysis. Then density gradient centrifugation on sucrose is performed and plasma membrane layer is aspirated. Since the Fc receptor is a transmembrane glycoprotein it can be solubilized with nonionic detergents such as Triton X 100 or Noniodet P-40. After solubilization of membrane the Ig receptor can be isolated by repetitive affinity chromatography. The ligands usually used in the isolation of Ig binding receptor are monomeric IgG, chemically cross linked IgG, heat aggregated IgG and anti Ig binding receptor antibodies. Usually Sepharose 4B has been used as solid matrix for immobilizing the immunoglobulins. The affinity gel is prepared from Sepharose 4B by chemically cross linking the ligands by standard procedure. The receptor bound to the affinity column is generally eluted with 0.5 N acetic acid containing 0.1% NP-40. However other reagents have also been used. These in the order of their effectiveness are 2% SDS > 6 M guanidine HCl >O 5% deoxycholate > 3 M KSCN > 3.5 M $MgCl_2$ > 8 M urea > 5 M KI > 2 M LiCl > 3 M KCl > 1 M

acetic Acid > 1 M propionic acid (Takacs, 1980). Less amount of receptor is obtained if NP-40 is not added in eluting buffer (Takacs 1980).

One of the usual contaminating protein is actin which can be removed by prewashing the affinity gel with low ionic strength buffer. This would cause depolymerization of actin and its elution from the column. Since the elution is not specific it would always be advisable to isolate Ig binding receptor by repetitive affinity chromatography.

Molecular Weight of Ig binding receptor

The reported values of molecular weight of Ig receptors differ widely and lie in the range 12-200 kDa. Molecular weights have generally been determined by SDS-PAGE, however in recent years the molecular weight of receptor on chicken erythrocytes has been determined by gel filtration. The values are listed in Table IV. The difference in the molecular weights of different receptors may represent receptor heterogeniety. Alternatively it may arise from the difference in the methods used in the isolation. Proteolytic breakdown of the parent molecule may partly be responsible for such discrepancy. It is quite possible that Ig binding receptor activities with different molecular weight may exist on a single cell. In

TABLE IV

Molecular weights reported for different IgG binding receptor

Cell line used	M.W. kDa	Reference
Human Platelete FcR	210	Stricker et al., 1987
Human Platelete FcR	200	Vancura and Steiner, 1987
Human T Cells	120	Cunningham Rundles et al., 1980
Rabbit lymphocytes	110-120	Sire et al., 1980
Murine Mastocytoma P815	115	Kahn Perles et al., 1980
Human monocytes U937, H660	70, 40	Frey and Eugelhardt, 1987
Human Neutrophil FcR	66, 53	Fleit et al., 1982
Human Eosnophil	43	Kulczycki, 1984
Suppressor Cell hybridoma	56, 61	Kulczycki et al., 1986
Guinea Pig Peritoneal Macrophages	44	Yagawa, et al., 1985
Platelete	40	Kelton et al., 1987
Chronic Myeloid leukemia	42, 18	Stein et al., 1981
B Type leukemia	28	
Chicken Erythrocytes*	30	Manghi et al., 1987
Soluble Fc R T cell line KE37	23	Caraux et al., 1983(b)
Pig lymphocytes	20-18	Vojtiskova and Franek, 1984
Thymocytes	12	Bezvershenko et al., 1980(a)(b)

* Molecular weight determined by gel filtration analysis, for all others molecular weight determined by SDS PAGE.

such cases the affinity chromatography may not be a suitable technique for obtaining a pure and homogeneous receptor preparation.By using affinity columns of monoclonal antibody raised against Ig binding receptor it is possible to obtain a homogenous and pure preparation of the receptor. However the disadvantage of this method is that Ig binding receptor lacking the particular epitope against which monoclonal antibodies are directed may not be bound to the affinity column. The value of molecular weight as determined by SDS-PAGE should be taken with caution since the receptors are glycoprotein for which the molecular weight estimate by SDS-PAGE may represent an over-estimation (Shapiro et al,1967). In SDS-PAGE different values of molecular weight were found using different crosslinking of the acrylamide gel. Thus with 5.6% acrylamide gel Fc R from rabbit alveolar macrophage moved as two bands with molecular weights of 50 kDa and 70 kDa but in 9% gels the receptors moved as two bands with molecular weights of 35 kDa and 55 kDa. In gel filtration the elution volume of the protein correlates better with its Stokes radius rather than with the molecular weight. The molecular weight of the receptor as determined by gel filtration is likely to be in error because of the glycoprotein nature of the receptor(Waheed and Salahuddin,1975). The difference in molecular weight may arise from the difference in the extent of glycosylation (Green et al., 1985).

For example Fc R on P388D, J 774 and B cells moved as a 60 kDa and 64 kDa species in SDS-PAGE. After removal of carbohydrate by endoglycosidase F the receptor moved as a single 37 kDa band. (Green et al ,1985).The heterogeniety of Ig binding receptor may be due to the proteolytic fragmentation of the parent molecule as has been demonstrated by the work of Kahn Perles et al (1980) on Ig binding receptor expressed on P815 cells . In the absence of protease inhibitors the receptor moved in SDS-PAGE under reducing conditions as 90, 70, 45 and 23 kDa species. However, in the presence of inhibitor the receptor moved essentially as a single 110 kDa species. On the basis of these results Kahn Perles et al (1980) suggested domain structure of Ig binding receptor.

The Glycoprotein nature of Ig binding receptor

The glycoprotein nature of Ig binding receptor has been established (Takacs ,1980). It can interact with concanavalin A-Sepharose (Mellman and Unkeless,1980), can be labelled by galactose oxidase NaB($^{3}H_{4}$) procedure (Mellman and Unkeless, 1980) and by biosynthetic labelling using ^{14}C glucosamine (Kulczycki et al, 1980). The treatment of the receptor by neuraminidase leads to a shift in its isoelectric point to more basic pH values suggesting that the receptor is a sialoglycoprotein

(Mellman and Unkeless, 1980, Rosenfeld et al, 1985). The differential sialation leads to microheterogeniety on isoelectric focussing (Vaughan et al, 1985).On the human peripheral lymphocytes treated with neuramindase, sialic acid deprived $Fc_{\gamma}R$ are not repaired until the denovo resynthesis of receptor takes place (Katoaka et al, 1985). On chicken embryonic thymocytes and bursa cells neuraminidase treatment reveals additional receptor sites (Nowak et al., 1985). Ig binding receptor from mouse hybrid cell lines DCH5 and human placental have been shown to contain 34% (w/w) (Zikan et al, 1986) and 30% (w/w), (Mikul ska and Liswoski, 1985). carbohydrates respectively. Ig binding receptor shed from human peripheral mononuclear cells bound to concanavalin A,Pisum sativum, Ulex europeus and peanut lectins indicating the presence of glycosyl, mannosyl, fucosyl and galactosyl groups (Sandor et al, 1986). These groups did not interfere with the interaction between IgG , Fc and Ig receptors showing that they were not part of IgG binding site. At 10 mM concentration galactose and alpha methyl mannoside did not have any influence on suppression of antibody production by shed Ig binding receptor. However several lines of evidence implicate carbohydrates in some of the Ig binding receptor functions. IgE present on rat basophilic leukemia cells cultured in the presence of glucosidase I inhibitor shows significant decrease in

their capacity to bind mouse monoclonal IgE (Gav eriaux and Loor,1987).HLA DR1 and DR3 positive monocyte from normal individuals have impaired Fc gamma receptor mediated phagocytosis (Salmon and Kimberly, 1986). Also a decrease in phagocytosis of concanavalin A treated erythrocytes was observed. This raises a possibility that carbohydrate-lectin interaction might trigger ingestion mediated with Fc R. Modulation of Fc R by binding to solid phase IgG aggregates specifically reduces the internalization of concanavalin A and IgG sensitized erythrocytes. Modulation by solid phase concanavalin A had no effect on phagocytosis of any other particle. Alpha methylmannoside (0.1 M) had no effect on internalization on EA but blocked ingestion of concanavalin A sensitized erythrocytes by 97%. These results indicate that a mechanism for ingestion of concanavalin A treated erythrocytes integrally involves Ig binding receptor (Sal mon et al, 1987). This Ig binding receptor contains an oligosaccharide with an exposed mannose. This mannose does not play an important role in IgG Fc-Ig binding receptor interaction. However binding to the receptor via mannose can initiate internalization. These results indicate the possibility that nonimmune function may utilize these receptor through carbohydrate interactions.

Lipids Attached to Ig binding receptor

Fc γ R of human B cells has been found to contain phospholipids and fatty acids in the ratio of one is to one for lipid is to protein (Suzuki, 1983). The phospholipids are tightly bound and are not completely removed by treatment with chloroform methanol. Treatment of Fc R of the human B cell with phospholipase A_2 did not abolish its activity, however guinea pig macrophage receptor was inactivated by such treatment. Activity of delipidated receptor is restored on reconstitution with phospholipids(Aida and Onoue,1983).Phosphotidyl choline is the most effective in reconstitution .Phosphotidyl serine ,phosphotidyl inositol and sphingomyelin were ineffective(Itonaga et al ,1984).

Membrane Insertion of Ig binding receptor

The high affinity of the receptor for lipids coupled with its ability to partition in non aqueous phase in water detergent system are consistent with its membrane protein nature. The transmembrane nature of the Ig binding receptor can be deduced from experiments from $IgG_{2a/2b}$ binding receptor from murine macrophage (Green et al., 1985). Intact receptor had a molecular weight of 53 kDa which was precipitated with antibody raised against the receptor. After treating the cells with proteinase K, a protein was isolated from the cell membrane which could

cross react with anti 53 kDa antibody. These results showed that Fc R is synthesized as a 53 kDa protein which is inserted into the membrane as 38 kDa portion which is protected from digestion cleaving a 15 kDa fragment. It has been found that the 15 kDa external fragment contains four N linked oligosaccharide chains.

Amino acid sequence of Ig binding receptor

Amino acid sequence of Ig binding receptor isolated from 5 different sources have been deduced from the analysis of cDNA for the receptor. The sources include mouse macrophage cell line P388D1 (Lewis et al., 1986), murine macrophage cell line J774, T cell line S49.1 (Ravtech et al., 1986) and human NK cells (Simmons and Seed, 1988). The five amino-acid sequences are depicted in Figure 6 where, α, β_1 and β_2 are the three different genes for mouse macrophage Fc R. Alpha gene is expressed only in normal peritoneal macrophages and macrophage cell lines whereas β gene is expressed in lymphocytes as well as in macrophages (Ravtech et al., 1986) β_1 and β_2 genes are identical except for an insertion of 46 amino acids in the β_1 gene in the cytoplasmic region (Ravtech et al., 1986). The amino acid composition as determined from the nucleotide sequence are given in Table V. The β_1 receptor is the largest and the human NK cell receptor is the smallest of all the five receptors.

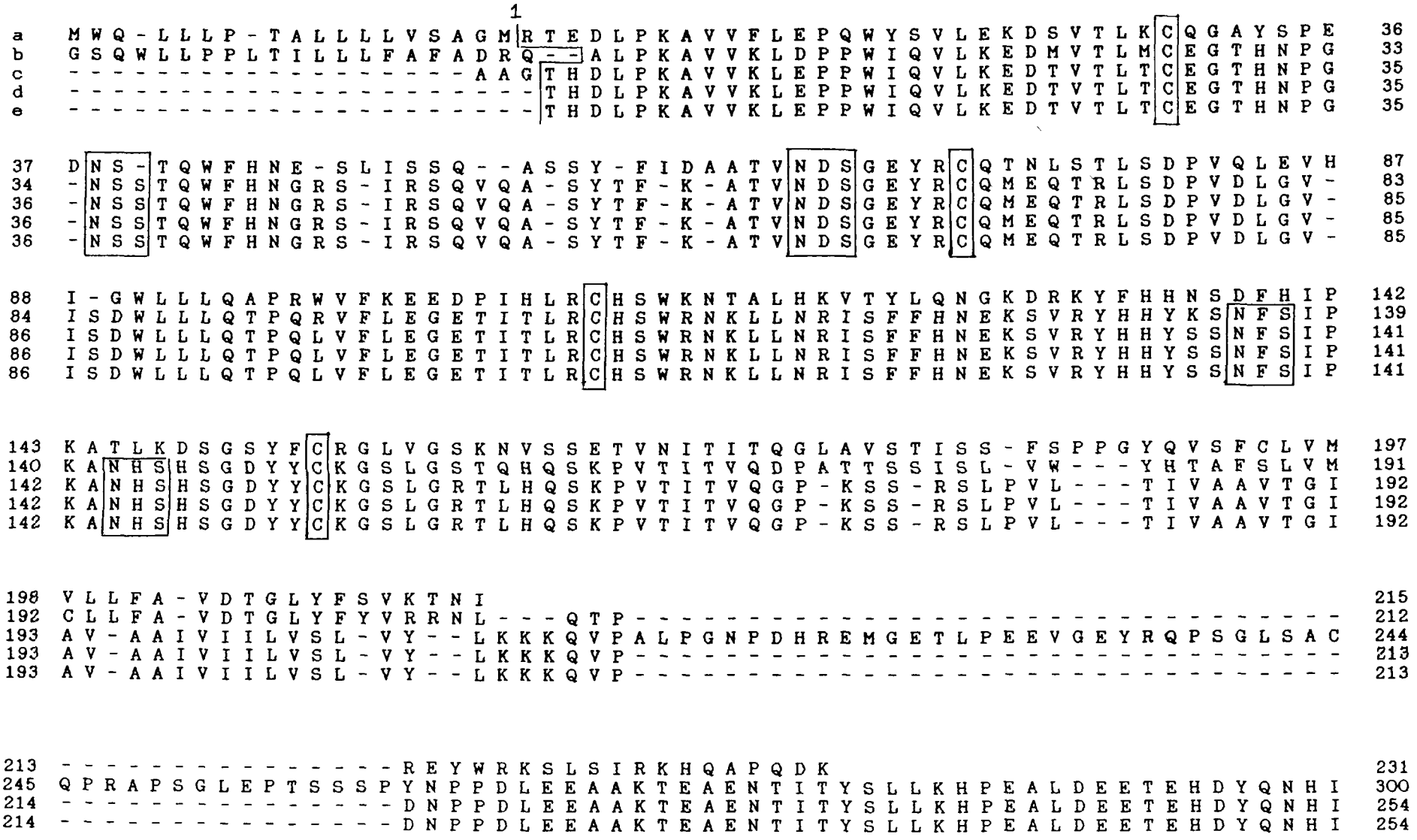


Figure 6.

Comparison of amino acid sequence deduced from nucleotide sequence for (a) human NK cells receptor (Simmons and Seed, 1988) (b) mouse macrophage FcR α_1 (Ravtech et al., 1986) (c) mouse macrophage FcR β_1 (Ravtech et al., 1986) (d) mouse macrophage cell β_2 (Ravtech et al., 1986) (e) mouse macrophage cell P388D$_1$ (Lewis et al., 1986). The number of amino acid residues is shown on both sides. The conserved cysteine residues and carbohydrate attachment sites are boxed.

TABLE V

Amino acid composition of FcR deduced from nucleotide sequence of various sources

	P388D1[a]	J774[b] α_1	S49.1[b] β_1	J774[b] β_2	NK
Ala	13	8	16	13	10
Arg	10	15	13	10	6
Asn	12	15	12	12	10
Asp	11	10	12	11	11
Cys	4	5	5	4	5
Glu	16	7	22	19	8
Gln	12	11	14	9	12
Gly	11	9	16	11	10
His	13	11	14	13	8
Ile	14	9	13	14	9
Leu	24	19	30	24	21
Lys	15	15	15	16	12
Met	1	4	2	1	1
Phe	6	10	6	17	12
Pro	14	10	22	13	8
Ser	23	22	28	22	25
Thr	21	18	24	22	15
Trp	5	7	4	4	5
Tyr	9	10	11	9	9
Val	20	16	21	20	18
Total	254	231	300	254	215

(a) Lewis et al., 1986

(b) Ravtechet al., 1986.

(c) Simmons and Seed, 1988.

The N terminal amino acid residues of the NK cell receptor and the α_1 receptor are arginine and alanine, respectively whereas all the three, β_1, β_2 and P388D1 receptors have threonine at the N terminal end. The NK cell receptors β_1 , β_2 and P388D1 cell receptors have isoleucine at the C terminal end. Only the α receptors has a lysine residue at its C terminal end. There is little variation in the composition of amino acids in these receptors. There are 4 tyrosines, 4-5 aspartic acids and 5-6 lysine residues per hundred residues in all the five Fc Rs.

Proline per hundred residues varies from 4 to 5.5 except for FcR from S49.1 T cell where the number is 7.3. The number of cystein residues interestingly is two per hundred amino acid residues in all the five receptors. If two cystine residues are involved in disulfide bond formation there would be one disulfide bond per 100 residues. Sequence analysis suggested an intrachain disulfide loop for every 42 to 45 amino acid residues. The distribution of acidic and basic amino acids for α form of receptor from J 774 cells is given in Figure 7. The amino acid sequence shows two clusters of basic amino acid residues, one from residues 106 to 133 containing 11 basic amino acid residues but only one acidic amino acid residue. The other, second cluster starts from residues

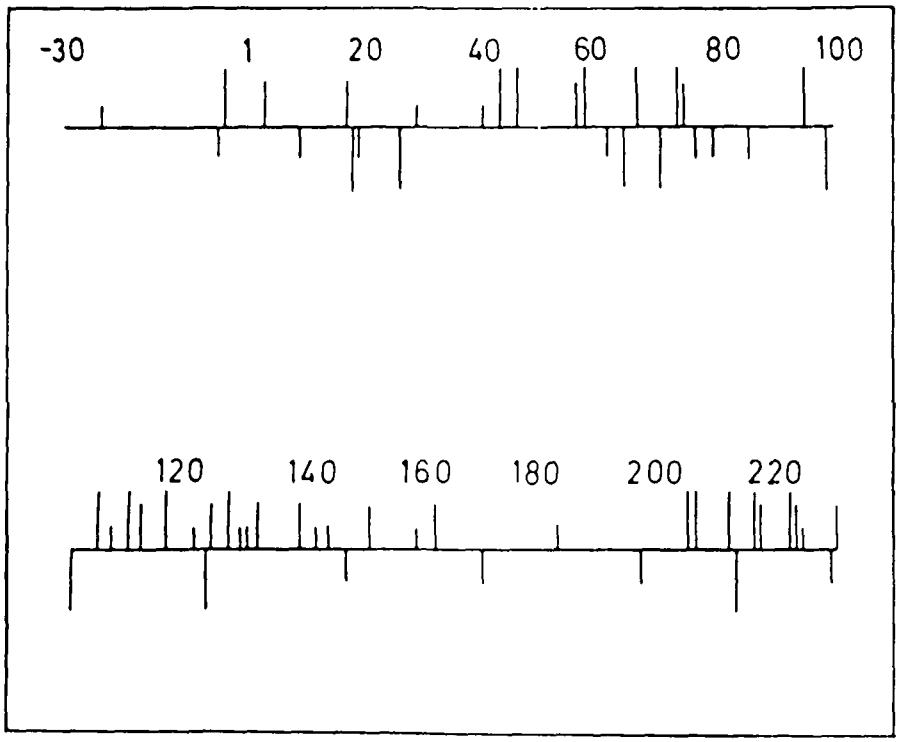


Figure 7

The distribution of acidic and basic amino acids of J774 cells FcR according to the amino acid sequence deduced from cDNA (Ravtech et al ,1986). Arginine (⊥) lysine (⊥), histidine (⊥), aspartic acid (⊤) glutamic acid (⊤).

206 to 231 with 10 basic amino acids residues and only two acidic amino acids residue (See Figure 7).

Schematic representation of the different regions of Fc γ R as deduced from amino acid sequence is given in Figure 8. All the receptors contain a signal peptide, an extra cellular region, a transmembrane region and a cytoplasmic region. The size of different receptors can be seen in Figure 8.

The signal peptide of P388D1 is smallest and those of β_2 and β_1 receptor are of similar size. Extracellular region is largest in NK cells followed by α_1 receptor. The extracellular region of β_1 and β_2 and P388D_1 cells are of almost similar size. The extracellular domain contains four potential N linked glycosylation sites. A serine and threonine rich region preceeds transmembrane region. The transmembrane region of α_1, β_1, β_2 and P388D, FcR are 20, 26, 26 and 29 residues long respectively. Analogus to the transmembrane region NK cells contain a hydrophobic stretch of 21 amino acid residues followed by four hydrophilic residues only one of which is charged. (Simmons and Seed, 1988). This terminal end has a glycosyl phosphatidyl inositol phospholipid (GPI-PC) moeity linked to it (Simmons and Seed, 1988). The extracellular domains of these receptors which are capable of binding immunoglobulins show considerable homology but

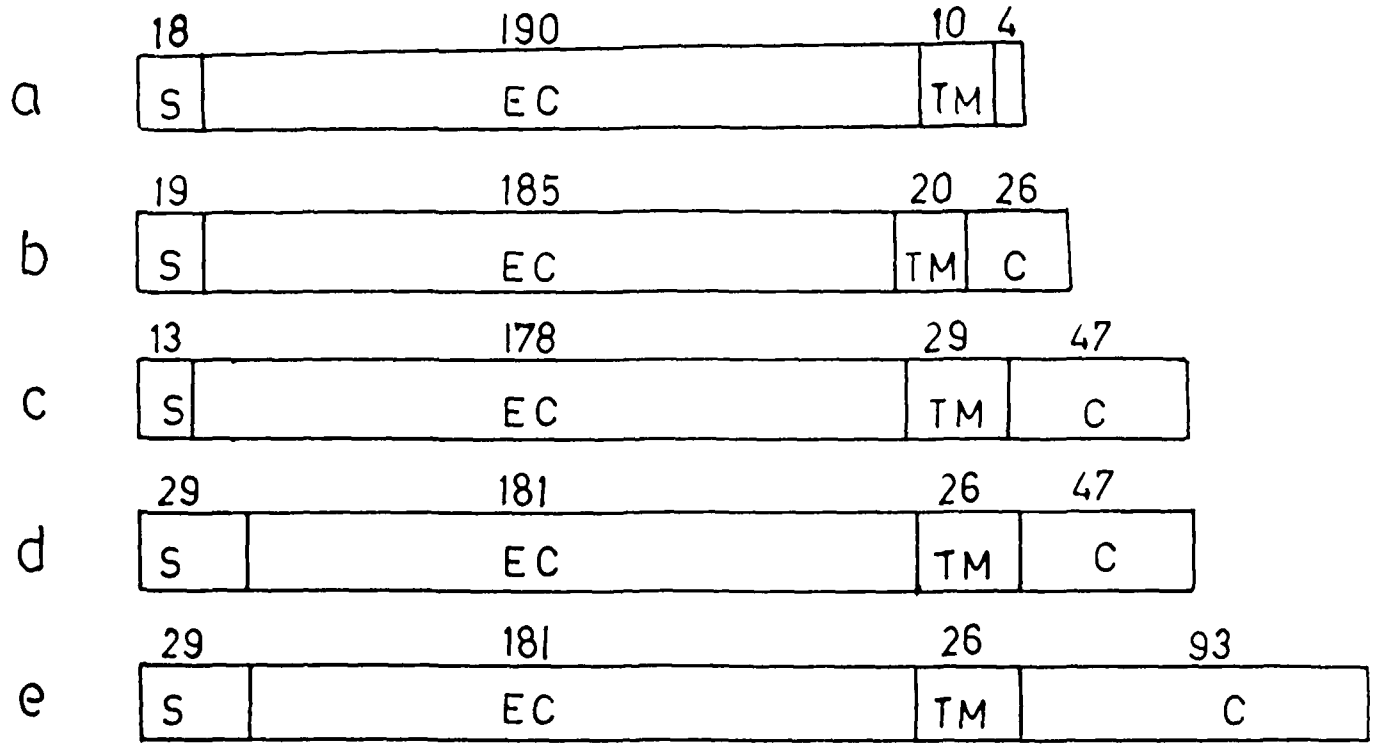


Figure 8

Summary of the structure of the FcR cDNAs of (a) human NK cells Fc R (Simmons and Seed,1988) (b) mouse macrophage FcR α_1 (Ravtech et al, 1986), (c) mouse macrophage P388D$_1$FcR (Lewis et al., 1986) (d) Mouse macrophage FcR β_2 (Ravtech et al.(1986)) (e) Mouse macrophage Fc$_\gamma$ R β_1 (Ravtech et al., 1986) S, the signal sequence, EC, extra cellular domain, TM the transmembrane domain, and C the cytoplasmic domain. Number of amino acid residues are shown on top.

differ in the cytoplasmic region indicating that the two receptors may transmit different signals after binding with the same extracellular ligand (Ravtech et al., 1986). It can be seen from Figure 6 that the extracellular segment from residues 1-120 of α_1 and β_1 and β_2 receptors exhibits about 100% sequence homology with respect to the sequence of $P388D_1$ receptor. The residues from 120 to 150 of $P388D_1$ cells receptors are 100% homologus to β_1 and β_2 receptors, however with receptor the homology is reduced to 76%. The residues from 181 to 210 corresponding to transmembrane region and 211 to 254 of $P388D_1$, β_1 and β_2 receptors are identical again showing 100% homology except for a 46 amino acid insertion at residues 214 in the β_1 gene. Interestingly the α_1 receptor shows a homology of 40% from residues 181 to 210 but only of 9% from residues 211 to 231 with respect to the sequence of $P388D_1$ receptor. The insertion in the β_1 gene may arise after alternate splicing of gene which may be regulated during macrophage maturation. The function of the inserted amino acid segement is not yet understood but it is possible that the longer cytoplasmic domain in β_1 FcR interacts differently with cytoplasmic or membrane proteins involved in signal transduction (Ravtech et al., 1986).

When compared to the sequence of the receptor the NK cell receptor showed around 36 to 80% sequence homology

extending from transmembrane to extracellular region. However the residues at the C terminal end were totally different. The comparison of the sequence of the extracellular region of α_1, β_1, β_2 and $P388D_1$ receptors reveal significant homology to immunoglobulin molecules. MHC class I and class II proteins, β_2 microglobulin, and other member of this supergene family (Hood et al., 1985, Ravtech et al.,1986, Lewis et al., 1986). The cluster of homology is centered about the cysteine residues in both the extracellular domain of FcR and kappa chain variable (V_K) region. The invariant residues of all K light chains are found conserved in each domain of FcR protein. A 32 percent identity over a stretch of 91 amino acids is found for FcR gene and MHC class II protein, $E\beta$ which itself contains an immunoglobulin like domain. (Ravtech et al., 1986). Similarly poly Ig receptor present on glandular epithelial cells involved in transepithelial transport of IgA and IgM was found to contain immunoglobulin like domains (Mostov et al., 1984).

A significant sequence homology was also found between FcR and an IgE binding protein (EBP) (Albrandt et al., 1987) from rat basophilic leukemia cells. Two regions, one with 38% identity over 26 amino acid residues and another with 25% identity over 52 amino acid residues have been determined. A very unusual homology was

found when the cDNA for human lymphocyte Fc receptors for IgE was sequenced(Ikuta et al ,1987). Out of 120 amino acid residues from the C terminal region 42 residues were identical with those of human and rat asialoglycoprotein receptor showing a 35% homology. In the same region chicken hepatic lectin and rat mannose binding protein had 39 and 27 residues identical to the IgE receptor sequence showing a homology of 33% and 23%, respectively. However no substantial homology was found with proteins from immunoglobulin gene superfamily.

Monomeric and Polymeric Forms of the Ig binding receptor:

Two forms of IgG binding receptor one which was shed after 4 to 37^{o}C (FcR I) temperature shift and another which is not shed (FcR II) under identical experimental conditions were detected on human peripheral lymphocytes (Sarmay et al., 1984). It was also proposed that the shed FcR may be freely exchanged between different types of human leukocytes and play a role in the suppression of mitogen induced T cell response. (Pitrech Noworolska et al., 1985). The shed FcR were found to be monomeric monovalent 60 kDa molecules possessing only one active binding site, interacting with the C_H3 domain of IgG Fc, while an additional binding site specific for C_H2 domain were found to be expressed on the polymeric crosslinked form. In the in vitro experiments crosslinking occurs

either by actomyosin (Uher and Jancso, 1981) or by transglutaminase (Fesus et al., 1982). Ca^{++} ionophore A23817 which is known to enhance transglutaminase activity, also induces receptor polymerization on cell surface. The polymerization was also termed as "receptor conversion" (Gergely et al, 1985) since it resulted in the expression of second binding site for the C_H2 domain besides the pre-existing C_H3 specific one. On the cell membrane also receptor conversion in both the directions was observed. Concanavalin A or interferon stimulation resulted in the expression of polymeric two binding site form whereas anti β_2 microglobulin antibodies induced shedding and depolymerization (Sarmay and Gergely, 1983). Anti β_2 microglobulin mediated changes were interrelated to activation stage of lymphocytes (Sarmay and Gergely, 1983). The fact that only polymeric form of the receptor is expressed on K and NK cells indicate that the receptor conversion reflects the prevailing stimuli as well as the actual level of cell differentiation.

Further investigation in this area reveals that lymphocytes involved in ADCC possess FcR having two binding sites whereas the interaction of both of them with Fc region is essential prerequisite of killing. One binding site reacts with the region 274/lys - 301/Arg within the C_H2 domain and the other interacts with the

region 408/Ser/416/Arg of the C_H3 domain. The signal for killing is not transferred equally by both the binding sites. The experiments done by blocking one of the sites while binding to the other indicates that the target effector binding utilizes C_H3 domains specific binding sites and the transfer of killing signal utilize C_H2 specific binding sites (Sarmay et al., 1985).

The assumption that such receptor conversion or polymerization is characteristic only for the K cell receptor can not be made. In phosphate buffered saline, the mouse macrophage Ig binding receptor forms large aggregates which retain Ig binding receptor activity but with altered IgG subclass specificity (Mellman and Unkeless, 1980). Various instances of Ig binding receptor existing in the polymeric or dimeric forms has been reported in the literature, functional importance of which is unknown. Human placental membrane Ig binding receptor is composed of 25-30 kDa chains which show IgG binding activity. These chains dimerize by disulfide linkage to form a 50-60 kDa subunit. Higher polymeric forms of this 60 kDa subunits were also found (Mikulska et al, 1982). Rat intestinal Ig binding receptor consists of 41-50 kDa protein which exists in the form of 100-110 kDa dimer. A 15 kDa protein is also associated with it (Simister and Rees, 1985). Ig binding receptor from human T cell line KE

37 is composed of 23 kDa subunits. Soluble form of the receptor is a dimer of 42-45 kDa. Higher molecular weight polymers of the 23 kDa polypeptide were also present (Caraux et al, 1983a). Janusz et al (1983) reported that guinea pig peritoneal macrophage Ig binding receptor exist in a 50 kDa form which is dissociated into a 25 kDa form. Ig binding receptor isolated from mouse macrophages cell line DC H5 (Zikan et al, 1986), from human serum (Sandilands et al, 1984), human T cells (Cunningham Rundles et al., 1980) and human lymphoblastoid cells (Takacs, 1980) are also reported to exist in the polymerized form. Cross linking of four Bcell Ig receptors is necessary for the transmission of inhibitory signals (Dickler and Kubicek,1988).

Ig binding receptor may act as a regulatory molecules at various levels of immune response. Different pathways of signal transfer and regulation can be initiated by Ig binding receptor depending on their primary structure, their actual conformation and interaction with other membrane components. In this context the phenomenon of receptor conversion is of great importance. The receptor conversion takes place most probably due to conformational alterations which results in the modification of binding sites as well. It is quite possible that the monomeric and the polymeric form of the receptor may induce different signals (Gergely et al., 1985).

Soluble Ig binding receptor:

Ig binding receptor like material, specifically binding to Fc fragment of immunolgobulin were found to be secreted or shed by T cells. These were called immunoglobulin binding factor (IgG-BF) (Rabourdin Combe et al., 1983). These have been found to be specific for IgG and IgE. Molecules having similar activities have also been named as soluble Ig binding receptor, Ig binding receptor like material, Fc binding component, EA agglutinating factor, etc. (Fridman et al, 1981). Whether IgG-BF and membrane bound Fc R are identical or not is yet to be established. IgG-BF is produced from Ig binding receptor$^+$ T cells and T cell lines and not by $Fc_\gamma R^-$ T cells. (Rabourdin Combe et al., 1983). These cells act as a non-specific suppressor cells. The suppressive activity of the cells is lost when the Ig binding receptor has been released from the membrane. This activity is found associated with IgG-BF. Production of IgG by B cell hybridoma and proliferation of these cells has been shown to be inhibited by IgG BF produced by T cell and its hybridoma T2D4 (Brunati et al, 1988). IgG-BF production is related to disappearance of membrane bound Ig binding receptor. Finally the antimacrophage Fc gamma 1/gamma 2b R monoclonal antibody 2.4G2 was found to bind to both the T cells Ig binding receptor as well as the IgG BF indicating that they share a common antigenic determinant (Daleron et

al, 1986, Blank et al.,1989). All these facts strongly suggest that the IgG-BF are derived from the cell surface Ig binding receptor, however they do not exclude the possibility that some IgG-BF may also be secreted without being first anchored in the membrane. Several monoclonal antibodies were also found to bind both, surface IgE receptor and IgE-BF. Peptide mapping of purified IgE-BF and Ig binding receptor also reveal several identical fragments indicating that IgE-BF is dervied from IgE Ig binding receptor (Nakajima et al., 1987).

Whether IgG-BF is generated by secretion and shedding or it is the product of proteolytic digestion of the membrane bound receptor remains unclear to date.

Digestibility of the protein molecule is highly affected by changes in the conformation of the protein. Activated T cells express receptor in a crosslinked form. IgG-BF may be formed by proteolytic clevage of crosslinked form of the receptor since cellular proteases are also present on activated T cells. (Gergely et al., 1985). Human peripheral blood lymphocytes Ig binding receptor are released from the membrane when incubated at 37^{o}C in serum free medium. Stage 1 occurs within the first hours of incubation and is probably mediated by limited proteolysis at cell surface. Stage 2 occurs between 2 and 4 hours and require active synthesis of Ig binding receptor. The

spontaneous release of Ig binding receptor was completely inhibited by the presence of serum. The serum factors containing alpha 2 microglobulin, and alpha 1 proteinase inhibitor were responsible for inhibition of Ig binding receptor release. (McGuire and Sandilands 1987).

Ig binding receptor interacts with a number of cytoskeletal elements like actinomycin complex, actin-heavy meromyosin and actin subfragments (Uher and Jancso, 1981). Binding of IgE receptor complex by polyclonal anti-IgE produces a detergent resistant network with cellular cytoskeletal elements (Roberstson et al., 1986). A number of contradictory reports have appeared in literature regarding the influence of various effectors on Ig binding receptor cytoskeletal interactions. Different Ig binding receptor types possess different susceptibilities to the cytoskeletal disrupting agents. At low concentrations vinblastin sometimes stimulate the expression of Ig binding receptor (Fornusek and Vetvicka, 1984). In monocytes vinblastin and vincristine influenced internalization of immune complexes, cytochalasin B influenced binding also whereas colchicine had no effect (Fornusek and Vetvicka, 1984). In B lymphocytes the cap formation of Ig binding receptor was inhibited only when cytochalasin B and colchicine were used together. When used alone colchicine enhanced this phenomenon (Fornusek and Vetvicka, 1984).

The temperature induced shedding of Ig binding receptor may also be due to release of anchoring of Ig binding receptor from cytoskeletal membrane proteins (Gergely et al., 1985). FcRI is not anchored whereas FcRII is anchored to the cytoskeletal contractile proteins (Uher et al, 1981). Interaction of monocytes bearing FcR1 with actinomycin induces a FcRI $\rightleftharpoons$ FcRII transition (Uher and Jancso, 1981). After temperature induced shedding the reappearance of FcR on cell membrane is not inhibited by protein synthesis inhibitor, cycloheximide, suggesting that the reexpressed FcR originates from a preformed receptor pool (Sarmay et al., 1980). However after antibody induced (β_2 Mi/anti β_2 Mi complex) shedding, FcR are not reexpressed on the cell surface.

Mechanism of Binding of Multivalent Immune Complexes/ Aggregated IgG to Ig binding receptor

Antibodies act as biological adaptors possessing the ability to link a large number of antigenic structures to a small number of immune effector system i.e. complement and cellular effectors through their Fc region. The total immunoglobulin concentration in blood is much higher than the concentration of immunoglobulins specifically bound to a particular antigen (Segal et al., 1983a). Receptors for IgG differ from other receptors (i.e. hormone receptors) by the fact that they must

recognize very low amount of antigen associated Fc fragment in the presence of excess amount of free immunoglobulins. Quantitative measurement of the binding of IgG to cells bearing FcR is done usually by using radiolabelled or fluoresceinated IgG oligomers (Segal et al., 1979).

FcR from trophoblasts bind monomeric and aggregated IgG with similar affinity (Burton, 1985). Monocytes bind aggregated IgG with similar or slightly higher (4 times) affinity than monomeric IgG. On the other hand neutrophils bind chemically aggregated IgG1 with 20 times greater affinity (Burton, 1985). Similarly platelets and lymphocytes are reported to bind aggregated IgG with higher affinity. The affinities of Ig binding receptor on various population of cells have been detected and are listed in Table VI.

The naturally occurring antigen antibody complexes are heterogenous in size and thus they cannot be used for studying the effect of size on oligomer binding. Bivalent affinity labelling reagents have been used to form IgG oligomers resembling immune complexes (Dower et al.,1981a). Aggregates analogus to antigen antibody complexes are also formed by heating IgG solutions.

The enhanced binding of aggregates/IgG immune complexes to Ig binding receptor have been explained by

TABLE VI

Association constants of various Ig receptors[a]

Cells	Immunoglobulin	Temp.	Kass.
Human Monocyte	Human pooled myeloma	37°C	IgG_1 $10^8 M^{-1}$
			IgG_2 $10^6 M^{-1}$
			IgG_3 $10^8 M^{-1}$
			IgG_4 $5x10^7 M^{-1}$
Human monocytes	Heat aggregated IgG	4°C	$10^{10} M^{-1}$
Mouse Macrophage like Cell Line P388D[1]	Mouse	37°C	$10^7 M^{-1}$
		4°C	$1x10^8 M^{-1}$
Rabbit Peritoneal Macrophages	Monomeric IgG	22°C	$6x10^5 M^{-1}$
Human PMNs*	Human myeloma IgG_1	4°C	dimer $5x10^5 M^{-1}$ oligomer $18x10^6 M^{-1}$

* Polymorphonuclear cells

(a) Burton, 1985.

proposing two different mechanisms. The first mechanism proposes an allosteric transition taking place after binding antigen to antibody. The transition passes from Fab region to Fc region causing an increase in the affinity of the Fc for the receptor. It was later observed that in many systems where antibodies have been polymerized by nonspecific methods such as heat aggregation or chemical cross linking, leaving the antigen combining site empty, bind the receptor with enhanced affinity. Also no conformational change has been detected by techniques including X rays (Amzel et al., 1974), NMR (Dower et al., 1977), limited proteolysis and reduction (Wright et al., 1978a) and the ability to bind protein A (Wright et al., 1978b). Monovalent haptens which are unable to polymerize antibodies cannot elicit effector responses. These lines of evidence thus discard the first mechanism.

The second mechanism proposes that enhancement in binding affinity arises because of the fact that IgG polymerized by various means (heat aggregation, reaction with multideterminant antigen, or chemically cross linked) are constrained to small volumes and thus present region of high Fc concentration to immune effectors (Segal et al., 1983b). High local concentration of both ligand and receptor favour enhanced binding. Once a complex has bound

to a receptor by one Fc region the binding of the remaining Fc region to free receptor is favoured. (See Figure 9). The second step occurs because Ig binding receptor are mobile on cell surface (Schreiner and Unanue, 1976) and IgG molecule are highly flexible (Cathou and Dorrington,1975). Binding constant increases with oligomer size because the large oligomers can bind with more subunits then the smaller ones. However very large aggregates (10 IgG molecules or more) bind less avidly than trimer because with the increase in the number of bound oligomer molecule there is a subsequent decrease of free receptor concentration. This makes in higher oligomers an increasing proportion of antibody molecules redundant. (Ratcliffe and Stanworth, 1983a).

The binding increases as the local concentration of Fc in immune complexes increases in relation to monomeric Fc in the medium. These in vitro studies indicate that in vivo total IgG levels in plasma may control most of the IgG mediated effector functions (Segal et al., 1983a). If the level of IgG in plasma falls, the magnitude of such effectors responses would increase.

The affinity with which the Ig binding receptor binds to multivalent immune complexes depends on the intrinsic affinity of a single receptor as well as cell surface

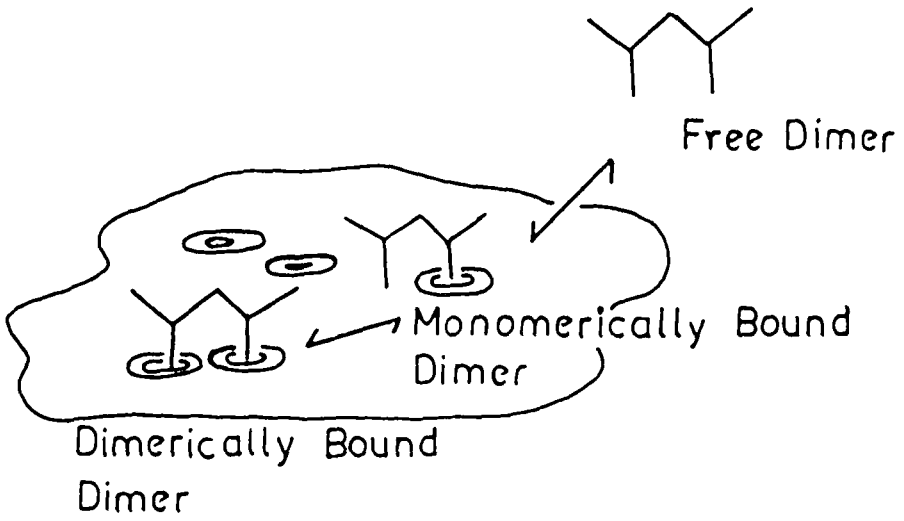


Figure 9

Schematic representation of the binding of an IgG dimer to Fc receptors. A molecule of solution phase dimer first binds to a free FcR on the cell surface. A free receptor then diffuses on the cell surface and forms a second bond giving rise to divalently bound species.

densities of the receptor. A cell therefore would modulate its affinity for immune complexes by varying its receptor density (Segal et al., 1983b). The affinity of cells for multivalent ligands can also be modulated by ligand self aggregation (Dower et al., 1981a). On studying the kinetics of association and dissociation of affinity cross linked IgG oligomers to $P388D_1$ cell surface Fc receptors it was found that monomeric IgG accelerates the rate of dissociation of bound oligomer from the cell (Dower et al., 1981b). Monovalently and divalently bound species are in rapid equilibrium with one another on the cell surface and the rate of formation of divalently bound species is faster than the dissociation of the monovalent species from the cell. The association reaction follows second order kinetics and the rate limiting step is the formation of the monovalently bound intermediate from the solution phase oligomer (Dower et al., 1981b).

The binding of human Fc to U937 cells is 3-4 times the rate of binding of human IgG 1 but it is also dissociated at about twice the rate. The association constants for Fc-FcR interaction and IgG FcR interaction were found to be $3.1 \times 10^9\ M^{-1}$ and $0.98 \times 10^9\ M^{-1}$ respectively (Raya Chaudhari et al., 1985). The binding of monomeric IgG_{2b} to rat macrophage was found to be temperature dependent as more IgG_{2b} bound to cells at $4^{\circ}C$ then at $37^{\circ}C$ (Denham et al., 1987).

Localization of Binding sites for Ig binding receptor on IgG molecules

Receptors on different cell types differ in terms of IgG binding constants, relative affinities of monomer and aggregated IgG and the domains of IgG molecules involved in this binding. Many investigators have tried to resolve whether it is $C_\gamma 2$, $C_\gamma 3$ or both domains of IgG which interact with Fc receptor under investigation. For these studies it is very important to consider homologus system (i.e. cell and IgG of the same species) and to consider each cell type individually with clear definition of aggregation state and subclass of the IgG involved. It may also be noted that heat aggregation of heterologous IgGs eliminate the species restriction on their binding to the lymphocyte receptors and these should not be used for determining Fc receptors specificity (Stout, 1981).

To locate the precise binding site two methods have generally been used. The first consists of preparation of fragments corresponding to the $C_\gamma 2$ and $C_\gamma 3$ domains and using these as competitive inhibitor in assays measuring FcR-IgG interactions. In the second method structurally abnormal variants of IgG molecules are used and the binding of FcR to these variant proteins is checked. Using these approaches the binding sites on IgG for the cell surface FcR have been studied for various heterogenous and homologous systems.

Human monocytes bind monomeric human IgG_1 and IgG_3 very tightly with a $K_{association}$ (37° C) of about 5 x 10^8 M^{-1} (Burton, 1985). By using monoclonal antibodies directed against epitopes on the $C_\gamma 1$, $C_\gamma 2$, $C_\gamma 3$ and $C_\gamma 2$-$C_\gamma 3$ interface it was found that $C_\gamma 2$ region of IgG plays major role in IgG monocyte FcR interaction (Partridge et al., 1986). Ratcliffe and Stanworth (1982) found that the peptides corresponding to residues 295-301 of the $C_\gamma 2$ domain (Aln-Tyr-Asp-Ser-Thr-Tyr-Arg) and 284-292 (Thr-lys-Pro-Arg) had weak inhibitory activity whereas dimeric $C_\gamma 2$ domain was capable of inhibiting IgG_1-human monocyte FcR interaction (Ratcliffe and Stanworth,1983b). C_H2 and C_H3 deleted paraproteins were used by Sarmay et al., (1986) to study the interaction between IgG_1 and FcR. These studies indicates that $C_\gamma 2$ domain plays significant role in the transfer of killing signal in ADCC by monocytes and that $C_\gamma 3$ domain is involved in high affinity binding to lymphocyte FcR.

In case of human polymorphonuclear leukocytes it is not yet clear whether $C_\gamma 2$ or $C_\gamma 3$ domain is involved in binding of IgG. However it appears that the site may overlap or be close to the protein A site (Burton, 1985)

Binding of rabbit IgG sensitized erythrocytes to human T cells was inhibited by human myeloma IgG. When the peptic fragment of Fc portion of IgG (pFc') containing $C_\gamma 3$

domain alone was used, the inhibition was higher as compared to $C_\gamma 2$ fragment indicating that $C_\gamma 3$ domain plays a major role in the binding (Burton, 1985). Reduction of inter heavy chain disulfide bonds of Fc has no significant effect on binding. But when another assay system involving binding of $Rh^+(D)$ antibodies coated erythrocytes to human lymphocytes FcR was used it was found that reduced and alkylated IgG lacked inhibitory ability (Klein et al., 1981). Reduction of the hinge region disulfides reduces the ability of IgG to bind to Fc receptor on human monocytes U937, M6O,ML-1 tumor cells, neutrophils, B cells, K cells, placental synctiotrophoblasts and murine macrophages (Dorrington and Klein, 1983; McCool et al., 1985). It is thus suggested that binding of IgG to FcR is sensitive to changes in the conformation of Fc upon reduction as well as in the degree of segmental motion of IgG. Similar behaviour is exhibited by hinge deleted proteins. Hinge deleted IgG binds to monocytes and aleveolar macrophages with reduced affinity (Burton, 1985).

On mouse macrophages are present Fc receptors of IgG_{2a} and IgG_{2b}/IgG_1. Subclass specificities seems to reside in the sequence of $Fc_{\gamma 2a}$ and $Fc_{\gamma 2b}$ since the Fc fragment inhibits only the binding of parent IgG to a cell surface receptor (Burton, 1985) Confusing results were obtained

when studies were done to localize the binding site on mouse IgG for mouse macrophage FcR. When the binding of labelled monomeric protein to mouse FcR was studied it was found that Fc fragments of IgG_{2a}, IgG_{2b} and IgG_1 were less inhibitory than their intact parent molecules respectively. The Fc fragment and pFc' fragment both had the same inhibitory capacity indicating that $C\gamma 3$ domain is essentially required for IgG_1 and IgG_{2b} binding to FcR (Burton, 1985). Ratcliffe and Stanworth (1983b) also recognized the major role of $C\gamma 2$ in IgG_1 binding to mouse macrophages. Hinge deleted IgG_1 inhibited the binding of IgG_{2b} to macrophage FcR but the binding of IgG_{2a} to the receptor remains unaltered. This implies the significance of $C\gamma 3$ in IgG_{2b} binding (Dorrington and Klein, 1983). Contradictory results were obtained when M 3.11 protein (IgG_{2b} with a deleted $C\gamma 3$ region) was used. This protein was equally effective as normal IgG_{2b} in inhibition of rosette formation between mouse macrophages and IgG_{2b} sensitized erythrocytes (Diamond et al, 1979). This result at variance with above mentioned results shows that $C\gamma 3$ domain is not critical in IgG_{2b} binding. Another variant protein used in these studies is ICR 1.6 which contains $C\gamma 1$ domain of IgG_{2b} and hinge, $C\gamma 2$ and $C\gamma 3$ domain of IgG_{2a} (Birshtein et al., 1982). Binding of IgG_{2a} to macrophages was not inhibited with intact ICR16 protein but with Fc fragment of the protein. These results suggest the Fab

fragments can influence the functional site on the Fc region either by inducing conformational changes or by masking relevant sequence. Diamond et al., (1985) have used cyanogen bromide fragment for the localisation of binding sites on IgG_{2b} and IgG_{2a}. There studies report that IgG_{2b} binds to 2b Fc receptor through a sequence in C_H2 domain and IgG_{2a} binds both C_H2 and C_H3 domains. It is also indicated that the binding of sequences from the C_H3 domain may induce a conformational change in the gamma 2a Fc receptor leading to enhanced binding of sequences from the C_H2 domain.

Carbohydrate chains attached to antibody molecules seem to play an important role in IgG-FcR interaction. Binding constant of monomeric IgG_{2a} to human monocytes decreased by 50% after aglycosylation(Leatherbarrow et al, 1985). Carbohydrate depleted mouse IgG_{2b} was prepared by treating antibody producing cells with tunicamycin (Nose and Wigzell, 1983). The carbohydrate deficient antibodies exhibited normal antigen binding capacity and protein A binding ability but lost the capacity to bind the FcR on macrophages, to induce antibody dependent cellular cytotoxicity and to activate complement. Human IgG monocyte interaction was found to be dependent on accessible galactosyl and mannosyl residues in the Fc domain (Malaise et al., 1987). It is proposed that this

lack of interaction of aglycosylated IgG to FcR may result either from a localized conformational change occurring in a single C_H2 domain or loss of conformation of C_H2-C_H2 domain which is stabilized by opposing and interacting oligosaccharide chains (Leather barrow et al., 1985).

Physiological Function associated with Ig binding receptor

(a) Role of Ig binding receptor in phagocytosis and antibody dependent cell mediated cytotoxicity

Ig binding receptor were found to mediate endocytosis of antibody opsonized liposomes by phagocytic ($P388D_1$) and non-phagocytic (P388) cell lines (Lesserman et al., 1980). Mouse macrophage Ig binding receptor for IgG1/IgG2 mediated the binding and pinocytic uptake of soluble IgG, and antibody-antigen complexes. Normal pathway of receptor mediated endocytosis i.e., internalization in clathrin coated pits and coated vesicles, delivery to endosomes and finally to acid hydrolase rich lysosomes was followed. The factors governing the rate of FcR mediated internalization of immune complexes include (a) the size of the complex, (b) the multivalency by which it binds to FcR (e) the mean life time a complex remains on the cell surface subsequent to binding (Segal et al., 1983c). Internalization of these multivalent IgG complexes was accompained not only by the

intracellular degradation of the ligand but also by a net decrease in the number of plasma membrane Fc receptor and an accelerated rate of membrane receptor turnover. Monovalent FcR-Fab complexes were internalized, delivered to endosomes and rapidly returned to the cell surface whereas polyvalent IgG-FcR complexes were transported to lysosomes and degraded.It appears that the receptor aggregated by multivalent ligands governs its fate on internalization.

Target cell coated with IgG can be killed nonspecifically through an extracellular nonphagocytic mechanism, involving non-sensitized effector cells which bind to the target cells by their specific receptor for IgG-Fc. Both phagocytic and non phagocytic myeloid cells (polymorps and monocytes) and K cells exhibit such antibody dependent cell mediated cytotoxicity. Inhibition of ADCC by low levels of aggregated IgG and antigen antibody complexes indicate the essential requirement of FcR binding in ADCC.

(b) Activation of adenylate cyclase

$Fc_{\gamma 2a}R$ and $Fc_{\gamma 2b}R$ present on the surface of $P388D_1$ cells after binding to specific ligand trigger the activation of adenylate cyclase system resulting in about six to seven fold increase in the accumulation of cellular cAMP (Nitta and Suzuki, 1982). $Fc_{\gamma 2a}R$ and $Fc_{\gamma 2b}R$ both

appear to activate adenylate cyclase by different mechanisms. The protein kinase activity associated with the IgG_{2a} receptor participates in the activation process(Hirata and Suzuki, 1987, Fernandez- Bortan and Suzuki,1986) The exact mechanism by which the $Fc\gamma_{2a}R$ mediated activation of adenylate cyclase occurs is not known. Another activity which has been detected by $Fc\gamma_{2b}R$ preparation is phospholipase A_2 activity which increases upon the binding of immune complexes(Hirata et al,1987) This activity results in the release of unsaturated fatty acids particularly arachadonic acid which is precursor of prostaglandins. The binding of prostaglandins to its specific substrate causes the activation of adenylate cyclase.

(c) Role of FcR in the regulation of immune response

Passive administration of antibody against an antigen results in the inhibition of specific immune response of the antibody against that antigen. Binding of IgG to IgG secreting B cells directly suppresses IgG production (Sherr et al, 1989). Further it was found that the Fc portion of the antibody was necessary for such inhibition to take place. The inactivation of B lymphocytes by FcR was given by the tripartite inactivation model which proposes that antigen antibody complexes interact with antigen sensitive cells when a determinant binds to the

antigen receptor and the Fc portion of antibody binds to the FcR. Thus a cross linking of FcR and antigen receptor takes place which inactivates immunocompetent cells as shown in Fig.10 (Sinclair, 1983).

The need for Fc region for crosslinking was confirmed by the fact that if only $F(ab_2')$ fragment of the antibody is added then B cell inactivation does not take place. But if antibodies against initial $F(ab_2')$ is added then inactivation does occur. The murine B cells are induced to proliferate after binding of (Fab_2') fragments of rabbit antimouse IgG to the cell surface. This $F(ab_2')$ anti IgG binding was also found to stimulate the rapid breakdown of inositol phospholipids resulting in the prolonged release of inositol polyphosphates and daicylglycerol (Bijsterbosch and Klaus 1985). However when intact antibodies were used instead of $F(ab_2)'$ then this response was abolished after 30 seconds. By blocking of cell surface Fc receptors or the Fc portion of the antibodies this inhibitory effect of intact antibodies is reversed. These results indicate that ligation of antigen receptor leading to cross linking of Fc and surface IgG receptor on B cells inhibit inositol phospholipids breakdown. As inositol phospholipid breakdown has been implicated in the induction of cell growth this effect may explain the inhibition of B lymphocyte activation upon binding of

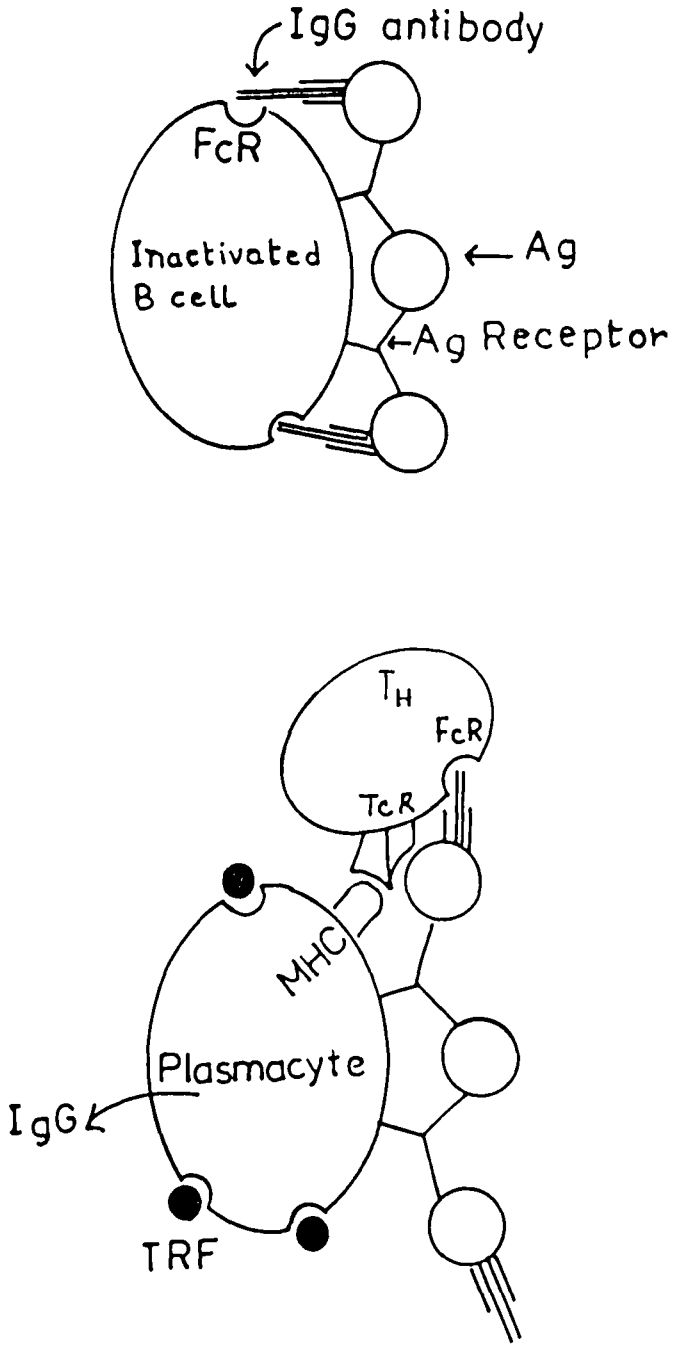


Figure 10

(a) The inactivation complexes made up of antigen, antibody and specific B cells and referred to as the tripartite inactivation model.

(b) Complete masking of plasmacyte FcR by TRF allowing the synthesis and release of large quantities of potentially suppressive IgG antibodies.

immune complexes. T cells appear to be resistant to Fc dependent antibody mediated immuno-suppression. Activated T helper cells bind to B cells in the context of B cell MHC antigen. The attached monomeric or pentameric antibodies are also recognized by T cells through their FcR (Dickler, 1983). These interactions between T and B cells result in the synthesis of antigen non specific T cell replacing factor, TRF (Sinclair, 1983), which interacts with B lymphoblast FcR. Binding of TRF to FcR might be a positive stimulus or prevention of negative stimulus. As FcR site is blocked by TRF no cross-linking with antigen receptor and hence no inactivation of B cells can take place. If antibody was added first, then TRF was without effect while if TRF was added first the addition of suppressive antibody was less inhibitory. These results suggest that suppression of immune response by endogeneously formed antibody might be prevented by TRF (Sinclair, 1983).

Miscellaneous functions implicated with Ig binding receptor

It was found that IgG_{2b}/IgG_1 FcR of J77 4 macrophage cell line functions as a ligand dependent ionophore (Young et al., 1983,a,b). The ionophore does not discriminate between Na+ and K+ and show low permeability to Ca^{2+}. However the Na^+/K^+ fluxes did not effect the rate of

phagocytosis, phagosomal acidification or superoxide anion generation (Pfefferkon, 1984). Human monocyte $Fc_{\gamma}R$ had been implicated in tumor cell killings (Graziano and Fanger 1987). Ig binding receptor bearing accessory cells facilitates the activation of T cells by anti CD3 antibodies (Austyn et al., 1987). Fc portion of human IgG was found to be responsible for the stimulatory effect on rat adipocytes lipogenesis (Khokher and Dandona, 1983). It might be that there are Ig binding receptor on adipocytes which mediate such lipogenesis on mast cells and basophils. The cross-linking of Fc R causes the release of vasoactive amines such as histamin and a slow reacting substance of anaphylaxis (SRS-A) (Froese and Paraskevas, 1983).

Available data on IgG receptor obtained from different sources show that the different receptors differ in pH stability (Wallace and Rees,1980,Mellman and Unkeless, 1980, Simister and Rees, 1985) and structure (Ravtech et al., 1986, Lewis et al., 1986); their biological functions appear to depend on cell type (Dickler and Kubieck, 1988). Further,aggregation of IgG receptor has been shown to be considerably significant (Gergely et al., 1985). Despite known species and cell type differences in structure and function of IgG receptor, studies on receptor from species other than

human, murine and guinea pig have attracted little or no attention thus far. Further, functionally important self association of receptor in aqueous solvent is yet to be investigated systematically. We have therefore isolated Ig receptor from hitherto uninvestigated sources i.e. goat peripheral blood lymphocyte. The receptor has been characterized for its carbohydrate composition, molecular weight, hydrodynamic properties and for its pronounced tendency to undergo pH and temperature dependent aggregation in aqueous buffer devoid of detergents.

II. EXPERIMENTAL

A. MATERIALS

1. Proteins:

Bovine serum albumin (lot No.100F-0249), ovalbumin (lot No.105C 8022), chymotrypsinogen A (lot No.11C-8170), cytochrome C (lot No.09C-0088), pepsin (lot No.60F-8056), antihuman IgG peroxidase conjugate (lot No.75 F-88-34), and Protein A (lot No.105 F 6834) were purchased from Sigma Chemical Company, St Louis Mo, U.S.A. Concanavalin A was purchased from Hygro Chemicals Pvt. Ltd. Calcutta, India.

2. Reagents used in polyacrylamide gel electrophoresis:

Reagents used in polyacrylamide gel electrophoresis were acrylamide (E. Merck, Dramstadt, Germany), N N' methylene-bis-acrylamide (Reanal Budapest, Hungary), N,N,N',N'- tetramethylethylenediamine (Ferak, Berlin, West Germany), bromophenol blue (BDH, Poole England) and glutaraldehyde (E. Merck, Dramstadt, Germany). Glycerol, acetic acid, formaldehyde and methanol were obtained from BDH, Bombay, India.

3. Chromatographic Media:

Diethyl aminoethyl (DEAE) cellulose (lot No.59C-0243) and Sepharose 4B were purchased from Sigma Chemical Company, St Louis, MO, U.S.A.. Blue Dextran and Sephadex G-200 were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

4. Reagents used in the isolation of lymphocytes and membrane proteins :

Histopaque-1077 (lot No.45-6174), Noniodet P-40 (lot No.103F-0594), phenyl methyl sulphonyl fluoride (lot No.103F-0493), and trypan blue were obtained from Sigma Chemical Company,St Louis, MO , U.S.A.. Other reagents used were iodoacetamide (Koch Light England), ethyelene diamine tetracetic acid (BDH, Bombay, India), ammonium chloride (E Merck, Dramstadt, Germany). Giemsa stain (S.D.S. Lab Chemical Industries, Bombay, India) and W.B.C. diluting fluid (Glaxo Laboratories, Bombay, India).

5. Miscellaneous reagents:

Other reagents used were orthophenylenediamine, adenosine monophosphate (lot No.109-C 7300), fluorescein isothiocyanate, isomer I (lot No.102 F 5045) from Sigma Chemical Company St Louis ,MO , U.S.A., hydrogen peroxide (Glaxo Laboratories, Bombay, India), sodium metaperiodate (Roanal, Budapest, Hungary), agar-agar (S.D. Fine Chem Pvt Ltd.,Boisar,India) phenol, sodium deoxycholate (Fluka, Switzerland), cyanogen bromide (Sisco Research Laboratories Pvt. Ltd. Bombay, India). HPLC grade methanol was purchased from S.D. Fine Chemicals, Bombay, India. Flat bottomed ELISA plates were purchased from Laxbro, Bombay, India.

All the other chemicals were of research grade and were used without further purification.

B. Methods

1. Preparation of solutions:

(a) Preparation of Giemsa stain:

For preparing the working solution of Giemsa stain first the following solutions were made

Solution A

Giemsa stain solution was obtained from SDS Lab. Chemical Industries, Bombay. The solution is normally prepared by dissolving 0.5 g of Giemsa stain dry powder in 33 ml of glycerine by heating at 50^{o}-60^{o}C for 2 hours in a water bath. Then 33 ml of acetone free methyl alcohol is added.

Solution B

This was prepared by mixing 722.2 ml of 0.15 M Na_2HPO_4, 77.8 ml of 0.15 M KH_2PO_4 and 1800 ml of distilled water. The pH of the solution was measured to be 7.0.

Working solution of Giemsa stain was prepared by mixing 1 ml of solution A and 2 ml of solution B and 47 ml of distilled water. Fresh working solution was prepared for each staining.

(b) Preparation of Folin-phenol reagent:

The Folin-phenol reagent was prepared by the method of Folin and Ciocalteu (1927). Hundred grams of sodium tungstate, 25 g of sodium molybdate, 700 ml of distilled water, 48.2 ml of orthophosphoric acid and 100 ml of concentrated hydrochloric acid were mixed together and the contents were refluxed gently in a round bottom flask covered with black paper for 10 hours. After cooling, 128.88 g of lithium sulphate, 50 ml of distilled water and a few drops of liquid bromide solution were added. The mixture was boiled for 15 minutes to remove excess bromine. The solution was cooled, filtered and diluted to 1.0 litre. The yellow coloured reagent was stored in brown coloured bottle. The above reagent was diluted 5 times with distilled water before use.

(c) Preparation of copper reagent:

The copper reagent was prepared by mixing 4% sodium carbonate, 4% sodium potassium tartarate and 2% copper sulphate in the ratio of 100:1:1.

(d) Preparation of Coomassie Brilliant Blue stain

The staining solution was prepared by dissolving 1 g of Coomassie Brilliant Blue R in 237 ml of methanol. To this solution was added 50 ml of acetic acid and 212 ml of

distilled water. The gel slabs were kept for overnight in this solution and then destained mechanically with 10% acetic acid.

(e) Preparation of solutions for silver stain:

Solutions for silver stain were prepared according to Oaklay et al (1980).

Fixative

A. 50% methanol (v/v) 7.5% acetic acid (v/v)

B. 5% methanol (v/v) 7.5% acetic acid (v/v)

C. 5% glutaraldehyde (v/v)

Diamine Silver Stain

In order to make 250 ml of the stain solution 53 ml of 0.09 M NaOH was mixed with 3.5 ml of ammonia solution (25%). To this solution was added dropwise 8 ml of 20% freshly prepared silver nitrate solution with gentle stirring. The volume was made up to 250 ml with glass distilled water.

Developer solution

This solution was prepared by mixing 250 ml of 0.05 % (w/v) citric acid with 1.25 ml of formaldehyde (37% v/v)

(f) Preparation of affinity columns:

Specific proteins were linked to Sepharose 4B for the preparation of affinity gels by the method of Kulczycki

(1983). For covalent attachment of protein to Sepharose 4B, the gel was activated with CNBr (300 mg CNBr/ml of gel) at 4°C adjusting the pH to pH 11 by adding small amount of 4M NaOH. After the CNBr was completely dissolved and the pH was stable the gel was washed extensively first with chilled water to remove excess of CNBr and then with chilled coupling buffer (0.2M sodium carbonate pH 8.2 containing 0.15M NaCl) . The protein to be coupled (1 to 2% solution) was taken in the coupling buffer and added to the activated gel and incubated at 4°C for two days. The gel was then washed and protein concentration was determined in the washings to calculate the unbound protein. The gel was then incubated with 0.2 M glycine in coupling buffer to block the free activated groups. The gel was then further washed and stored in PBS containing 0.02% azide. About 90-95% of the protein (HA IgG, BSA) was coupled to Sepharose 4B by this method. Generally affinity gels with 7-10 mg protein/ml of gel were used in these studies.

2. pH measurements:

pH measurements were carried out on Elico digital pH meter(model L1-120) using combination electrode. The least count of the pH meter was 0.01 pH units. The pH meter was routinely calibrated with 0.05 M pottassium hydrogen pthalate buffer pH 4.0 in acidic pH range and with 0.01M sodium tetraborate buffer pH 9.2 in alkaline pH range.

3. Optical measurements:

Absorbance of the solution in the visible range was measured on Photochem Colorimeter ,model C-110. In the ultraviolet region light absorption measurements were made on single beam Cecil spectrophotometer (model CE 202) and on Cecil double beam spectrophotometer, model CE 594. Fluorescence measurements were performed on Shimadzu Spectrofluorophotometer model RF-540 using quartz cell of 1 cm pathlength. The slit used was 10 nm.

4. Determination of protein concentration:

(a) Lowry's method

Protein concentration was routinely determined by the method of Lowry et al (1951) using bovine serum albumin as the standard .Typically 1 ml of protein solution was mixed with 5 ml of copper reagent. After 10 minutes 1 ml of Folin phenol reagent was added and the solution was kept for 30 minutes for the develcpment of colour. The absorbance was read at 700 nm.

(b) Determination of protein concentration by modified Lowry's method.:

Protein concentration in presence of detergents was determined by modified Lowry's method as described by Cabib and Polacheck(1984).

To 1 ml of protein solution was added 0.1 ml of sodium deoxycholate and 0.1 ml of 100% trichloroacetic

acid. After shaking, the mixture was kept in ice for 45 minutes and centrifuged at 3000 rpm for 50 minutes. The precipitated protein was washed with chilled 20% TCA solution twice. The precipitate was then dissolved in 1 ml of 0.1 N NaOH and then 1 ml of copper reagent (3% Na_2CO_3 in 0.1 M NaOH, 4% sodium potassium tartarate and 2% copper sulfate in the ratio 9.6:0.2:0.2 by volume) was added. The solution was incubated at 37°C for 10 minutes and then 0.1 ml of Folin's reagent was added. The mixutre was incubated again at 37°C for 15 minutes. The colour intensity was read at 700 nm against blank on Cecil single beam spectrophotometer. The linear curve (Figure 11) was found to fit the equation

$$O.D_{700nm} = 0.0025\ \mu g \text{ of protein} + 0.029 \qquad (1)$$

Protein in aqueous buffer devoid of detergent was also estimated spectrophotometerically using the equation as given by Boyer(1982).

Protein concentration (mg/ml) = $1.55\ A_{280} - 0.76\ A_{260}$ where A_{280} and A_{260} were absorbance at 280 and 260 nm respectively.

5. Ion Exchange Chromatography:

Ion exchange chromatography was performed using DEAE resin. The resin was swollen in distilled water. It was

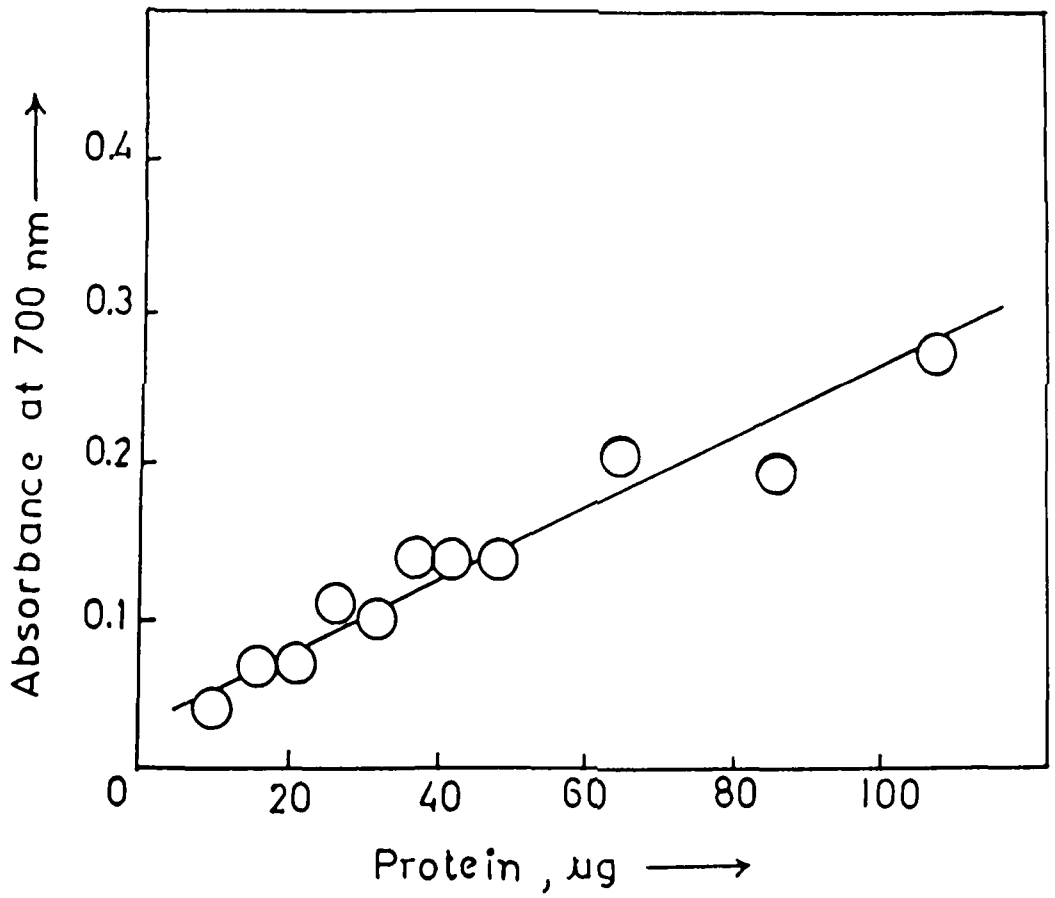


Figure 11

Calibration curve for the estimation of protein by th method of Cabib and Polacheck(1984) using bovine seru albumin as standard.

treated with 0.1 N NaOH for 1 hour ,washed with distilled water till pH becomes neutral, again treated with 0.1 N HCl for an hour and then washed with distilled water to remove excess of acid. It was packed in a column (2.2 x 15 cm) and equilibrated with the operating buffer.

6. Gel Filtration Chromatography:

Gel filtration experiments were performed on a Sephadex G-200 column. The column was packed according to the method of Ansari and Salahuddin (1973). About 10 g of gel was allowed to swell in distilled water for about 6 hours in an oven. The fine particles were then removed by decantation. This process was repeated several times until the gel was free from fine particles. A glass column (2.28 x 90 cm) was taken and washed with detergent, chromic acid and finally with distilled water. The column was then mounted in a vertical position with the help of clamps. Before packing the column its radius was determined at different positions along the height of the column. At three places 3 cm length of graph paper was pasted and the column was filled with distilled water. Volume of the water corresponding to 3 cm height were collected in three preweighed bottles. The volume of water was then determined by dividing the weight of water (w) by its density (d) at room temperature.

$$V = \pi r^2 l = \frac{w}{d}$$

$$\text{or} \qquad r = \sqrt{\frac{w}{d \pi l}} \qquad \ldots(3)$$

where r is the radius of the column. The radius of the column was thus determined to be 1.14 cm.

After determination of radius of the column, a small amount of glass wool, previously boiled in water, was placed at the bottom of the column with the help of glass rod. Few glass beads were placed on the surface of the glass wool. One third of the glass column was then filled with operating buffer keeping the outlet closed. An extension glass column (1.6 x 61 cms) was fitted on the top of the column and gel slurry was then poured slowly into the column through the extension with help of a glass rod. As the gel settled down the flow rate was increased gradually from 5 ml/hr to 40 ml/hr. The column was then equilibrated with operating buffer by passing a volume equal to three times of the total bed volume. Uniform packing of the column was checked by passing 0.5 % w/v solution of blue dextran.

The operating buffer on the top of the column was first drained off and 2-4 ml of the sample containing 30 to 60 mg protein was applied on the column with the help of an applicator. The stop cock of the column was then opened slowly and the sample was allowed to percolate

through the upper surface of the gel. As soon as all the sample entered the gel the column was rinsed with about 4 ml of the buffer solution. The flow rate of the column was then adjusted at 20 ml/hr and the column was connected to a reservoir containing operating buffer. After rejection of discarded volume, the protein fractions of 5 ml were collected and monitored by the method of Lowry et al., (1951).

7. High performance liquid chromatography:

The gel filtration chromatography of the immunoglobulin binding proteins was performed on HPLC Shim Pack Diol-150 column (0.79 x 25 cms). The flow rate of the column was maintained at 1 ml/min by Shimadzu LC-6A pump. The column was first washed with 4 column volumes of 20% (v/v) methanol followed by washing with 4 column volumes of 0.1 M NaCl. Always HPLC grade methanol was used. For the preparation of buffer recrystalized salts were used. The buffer was prepared fresh before use. The outlet and inlet tubes were always kept submerged in the solvents to prevent the entry of air bubbles in the column. All the samples were filtered through 0.45μm millipore filter before applying on the column. The protein peaks were detected spectrofluorometrically by Shimadzu RF-540 spectrofluorophotometer keeping excitation wavelength of 280 nm and recording emission at 354 nm. The slit width

was kept at 15 nm both for excitation and emission beams. The peaks were recorded automatically by C-R3A integrator. The equilibrating buffer did not show any fluorescence at these wavelength. The stability of the base line was checked for ten minutes before each analysis.

8. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis:

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was done according to the method of Laemmli (1970).Washed and siliconized slab was used.The gel was polymerized using 12.11% acrylamide, 0.33% methylene bis acrylamide, 0.05% N,N,N',N' tetramethylene diamine, 0.04% ammonium persulphate and 0.1% SDS in 0.4M tris HCl buffer pH 8.8 . Stacking gel was prepared using 4.35% acrylamide, 0.12% methylene-bis-acrylamide, 0.1% N,N,N',N' tetramethylene diamine, 0.03% ammonium persulphate and 0.1% SDS in 0.12 M tris HCl buffer pH 6.8. Electrophoresis was carried on in 0.025 M tris 0.192 M glycine buffer pH 8.3 with 0.1% SDS. Samples were prepared by dialysing the protein samples against 0.0625 M tris HCl buffer pH 6.7. They were made to 2% in SDS and heated for 10 minutes in boiling water bath. After cooling the samples (2 ml) were reduced by adding 0.05 ml of mercaptoethanol. A drop of glycerol and bromophenol blue solution was then added to the samples. Electrophoresis

was carried out for two to three hours by passing 50 m A current per slab (17x20 x 0.2cm) using a Systronic power supply 610.

The slabs were then stained by the following method. When 10 to 100 µg protein sample was applied on the gel , the slab was stained by commassie brilliant blue. The stain was prepared as described earlier. The slab was kept for overnight in this solution and then destained with 10% acetic acid. When low quantity of protein (less than 10 µg) was used in SDS PAGE the protein bands were stained by silver according to the method of Oaklay et al (1980). For silver staining the fixatives A, B and C, diamine silver stain and the developer were prepared as described earlier. The gel was fixed for one hour each in solution A, B and C. Then it was washed with large amount of glass distilled water (2-3 litres) for overnight. Before staining it was incubated at 50°C for one and a half hours to remove excess of glutaraldehyde which gives background staining (Allen et al., 1984). The gel was then stained in diamine silver stain for 20 minutes and then washed with distilled water for five minutes. The bands were developed by adding the developer. To control the background from darkening 1:1 diluted developer was used. After the bands were visible the slab was washed with distilled water to remove the developer and stored in 10% acetic acid.

9. Estimation of neutral hexoses and detection of sialic acid:

Neutral hexose content of the isolated protein was determined by the method of Dubois et al (1956) using mannose as the standard. The colour intensity at 490 nm was plotted against mannose concentration in micrograms. The linear curve(Figure 12) was found to fit the equation.

$$O.D._{490nm} = 3.95 \text{ milligram mannose} + 0.065 \quad ...(4)$$

To one ml of protein solution was added one ml of freshly prepared 5% w/v phenol solution in water followed by rapid addition of 5 ml of concentrated sulphuric acid. The hot tube was allowed to cool for 20 minutes at room temperature. The colour intensity was measured at 490 nm against appropriate blank prepared similarly by omitting the protein. The presence of sialic acid was detected by the method of Warren (1959). Protein was heated with 0.1 N sulphuric acid at 80^{o}C for 1 hour .After cooling 0.1 ml of 0.2 M sodium metaperiodate in 9 M orthophosphoric acid was added and the mixture was incubated for 20 min at room temperature .To this solution was added 1 ml of sodium arsinate in 0.5 M sodium sulfate containing 0.1 N sulfuric acid with vigorous shaking and the yellow colour which appeared momentarily disappeared .Then 3ml of sodium thiobarbituric acid in 0.5 M sodium sulphate was added.

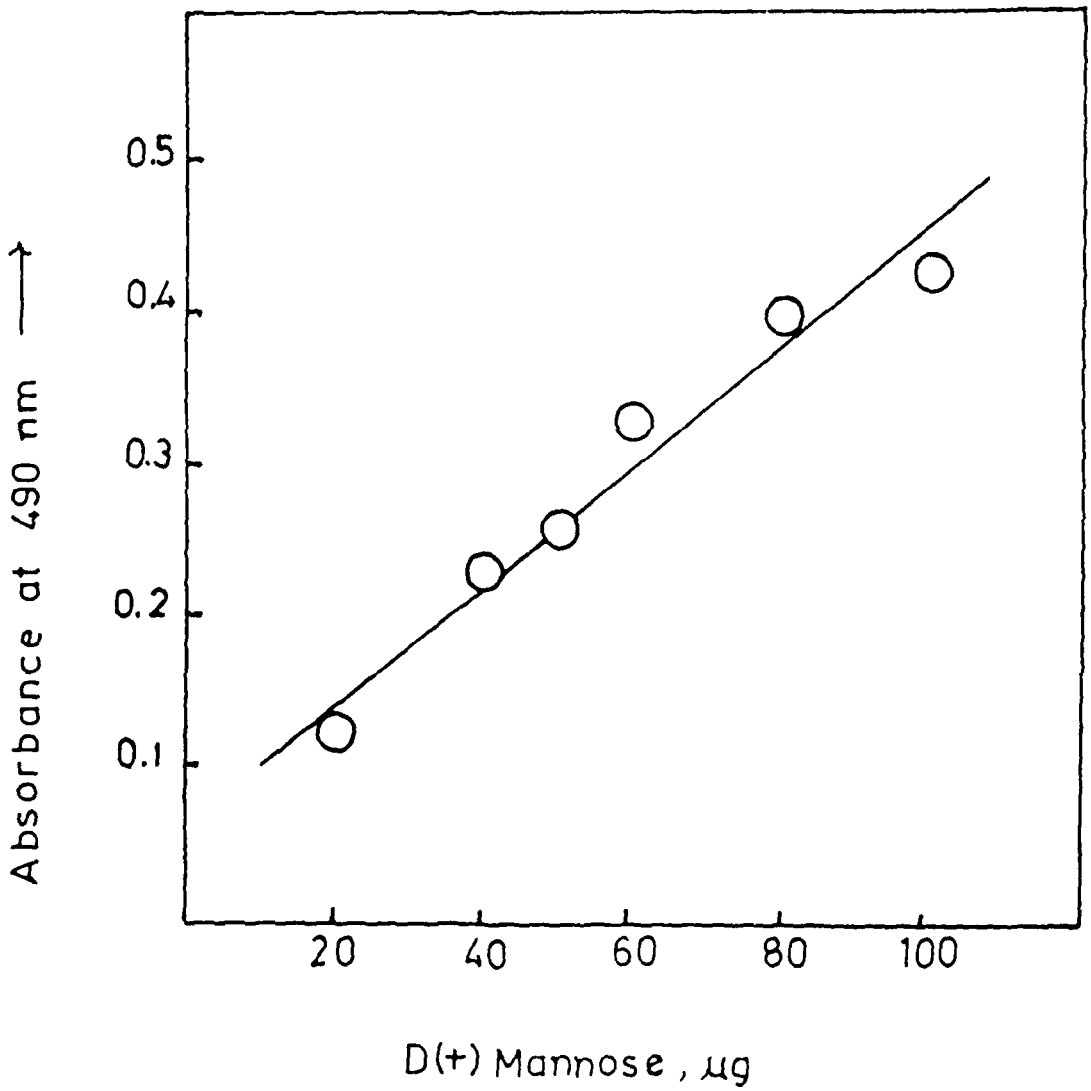


Figure 12

Standard curve for the estimation of neutral hexose by the method of Dubois et al, (1956).

This mixture was heated in boiling water bath for 15 min. It was cooled and diluted with an equal volume of cyclohexanone . The colour intensity of cyclohexanone layer was read at 550 nm. The solution prepared similarly with bovine serum albumin served as control.

10. Estimation of inorganic phosphate:

Inorganic phosphate was assayed by the method of Fiske and SubbaRow (1925) to measure the hydrolysis of AMP by 5'- nucleotidase. To 1 ml of solution containing phosphate was added 4 ml of double distilled water. To this 0.4 ml of perchloric acid (60%) was added. This was followed by the addition of 0.4 ml of 5% ammonium molybdate and 0.2 ml of 0.2% ascorbic acid. The tubes were heated for 2 minutes at 60^{o}C in a water bath and read at 600 nm after cooling.

11. Assay of 5' nucleotidase activity in membrane fractions:

For the assay of 5'nucleotidase activity the method of Jakoly and Morre (1971) was followed. The substrate consists of 11 mM AMP in 55 mM Tris HCl buffer pH 8.5,containing 5.5 mM magnesium chloride. To 0.5 ml of protein fraction was added 0.2 ml of 0.1 M sodium potassium tartarate and 0.4 ml of tris HCl buffer pH 8.5, followed by the addition of 0.9 ml of the substrate. The

samples were incubated for 1 hour at 37°C. The reaction was stopped with 2 ml of 10% TCA solution. After filtration 0.2 ml of filtrate was taken for the estimation of inorganic phosphate.

12. Isolation of immunoglobulins and its derivatives:

Goat IgG was isolated from plasma by the method of Gray et al (1969) with some modifications. Goat blood was collected in 3.8% sodium citrate solution in the ratio of 4:1. Plasma was isolated by centrifugation of blood at 2000 rpm for 20 minutes on Remi R23 centrifuge. It was made 40% in ammonium sulphate and kept for 6 hours at room temperature. It was then centrifuged and the precipitate was dissolved in 0.01 M phosphate buffer pH 8.0 and dialysed against the same buffer to remove ammonium sulphate. It was then applied on DEAE cellulose column and the unbound protein was collected. The bound protein was eluted with 0.1M NaCl in the same buffer. The protein eluting in both bound and unbound fractions was further purified on Sephadex G-200 column. All these fractions were analysed by SDS-PAGE. The fraction purified on Sephadex G-200 column were found to be free of extraneous proteins.

Human IgG was isolated by the method of Fahey and Terry (1979). For the isolation of human IgG the human plasma was made 40% in ammonium sulphate and the

precipitate was obtained by centrifugation as described above. It was then dialysed in 0.01 M phosphate buffer pH 8.0 and applied on a DEAE ion exchange column. In this case only the unbound protein was collected. The isolated protein was further purified on a Sephadex G-200 column (2.28 x 80 cm).

Aggregated IgG was prepared by the method of Schreiber and Haimovich (1983). The protein solution (5-10 mg/ml) was heated at 63°C for 10 minutes in a water bath. The aggregated protein was isolated by gel filtration on a Sephadex G-200 column. It was eluted in void volume of the column. The yield of aggregated goat IgG was found to be 5.12%.

The fragment $F(ab_2')$ of human IgG was prepared by the method of Stanworth and Turner (1986). Human IgG (1-2%) solution was treated with pepsin in the ratio of 100:2 by weight for 7 hours at 37°C in 0.2 M sodium acetate buffer pH 4.5 containing 0.15 M sodium chloride. The reaction was stopped by raising the pH to 8 by adding solid tris. The $F(ab_2')$ was isolated and purified by repetitive gel filtration on a Sephadex G-200 column. On SDS-PAGE the purified fractions were found to be free from uncleaved IgG.

13. Isolation of lymphocytes from goat blood:

Lymphocytes from goat blood were isolated by density gradient method of Boyum (1984). Freshly collected goat

blood was centrifuged at 2000 rpm for 20 minutes on Remi R23 centrifuge and the buffy coat was collected. The buffy coat was washed three times with phosphate buffered saline (10 mM sodium phosphate pH 7.4, 0.15M NaCl, PBS) to remove cytophilic IgG (Alexander et al, 1978). The buffy coat was then diluted 1:1 with PBS and 8 ml of this was layered on 3 ml of Histopaque - 1077. This was centrifuged on Remi T8C centrifuge at 2000 rpm for 20 minutes. The white layer at the interface of Histopaque and PBS was aspirated with the help of pasteur pipette. The collected cells were then washed with 0.83% NH_4Cl to remove contaminating red blood cells. The lymphocytes were then washed with PBS containing 0.2% sodium azide to inhibit receptor endocytosis (Alexander et al, 1979).

After isolation the cells were counted using a haemocytometer (Mishell and Shiigi, 1980). The coverslip was placed on the haemocytometer and using a pasteur pipette, cell suspension was gently applied between the coverslip and the haemocytometer. All the cells in the four large squares were counted. Each square of the haemocytometer with coverslip in place, represents a total volume of 0.1 mm^3 or 10^{-4} cm^3. Since $1cm^3$ is equivalent to approximately 1 ml the subsequent cell concentration per ml was determined by the following equation.

Cells per ml = the average count per square x 10^4 ...(5)

If diluted suspension was used then the count was multiplied by the dilution factor. The viable white blood cells were quantified by staining cell population with trypan blue. The dye is taken up by non viable cells and is excluded by the viable cells.

To 0.5 ml of trypan blue solution (0.16% in normal saline) was added 0.5 ml of the cell suspension. The cells were loaded on a haemocytometer and the blue stained (dead) and unstained (viable) cells were counted separately as described above.

$$\text{Percent viable cells} = \frac{\text{number of viable cells}}{\text{total number of cells}} \times 100 \quad \ldots(6)$$

The total number of cells include both the dead and the viable cells.

For differential leukocyte count a thin smear of cell suspension was made on the slide, it was air dried and fixed in methyl alcohol for 10 minutes. The smear was then stained in working solution of Giemsa stain for 10 min as described above . It was washed with water, air dried and observed under microscope. For staining the smear of goat blood the slide was kept in Giemsa stain for 30 minutes.

14. Isolation of lymphocyte membrane:

For the isolation of lymphocyte membrane cells were subjected to hypotonic lysis (Coligan and Kindt, 1986). The cells were suspended in 10 mM tris HCl pH 7.0 containing 1 mM $MgCl_2$, 1 mM KCl and frozen by keeping in the referigerator for about one hour. The cells were then thawed and the pH increased to pH 7.5 by adding solid tris. The cells were then disintegrated in a homogenizer with a tight fitting pestle for 3 minutes. The unbroken cells, nucleus and cell debris were removed by centrifugation at 2500 rpm on Remi R23 centrifuge for 10 minutes. Mitochondria and endoplasmic reticulum fractions were pelleted by centrifugation at 13000 rpm on Remi K24 centrifuge. The supernatant obtained was then centrifuged at 50000 rpm on Beckman ultracentrifuge L8-55M for 1 hour to pellet the membrane. The membrane pellet was immediately solubilized in solubilizing buffer (PBS containing 0.5% NP-40, 2 mM PMSF, 10 mM iodoacetamide and 3 mM EDTA).

15. Detection of IgG binding protein on goat lymphocytes by the binding of FITC conjugated IgG:

The specific binding of immunoglobulin to goat lymphocytes was detected by fluorometeric assay following the method of Schreiber and Hainovich (1983). For the

preparation of FITC-IgG conjugate, immunoglobulin solution was first dialysed against 0.2 M sodium carbonate buffer pH 9.0. A fresh solution of FITC was prepared by dissolving it in ethanol in order to get a solution of concentration 2.5 mg/ml. To the IgG solution (5-10 mg/ml) in 0.2 M sodium carbonate buffer FITC solution was added keeping about 25 µg of FITC for each milligram of IgG. This was incubated for 30 minutes with constant shaking. The unconjugated FITC was seperated from FITC-IgG conjugates by passing the mixture on Sephadex G-25 (2x20 cm) column. To obtain a homogenous preparation of conjugates, the conjugated protein was applied on DEAE cellulose column (2.2 x 15 cm) equilibrated with 0 01 M sodium phosphate buffer pH 8.0. The bound protein was eluted batchwise with 0.01 M sodium phosphate buffer containing 0.1 M, 0.25 M and 0.5 M NaCl. The fractions were monitored by taking the absorbance at 280 and 492 nm. The protein concentration was calculated by the equation

Fluorescein conjugated IgG (mg/ml)

$$= \frac{A_{280nm} - 0.35 \times A_{492\ nm}}{1.4} \qquad \ldots(7)$$

where A_{280} and A_{492} are absorbance at 280 and 492 nm respectively.

The molar F/P ratio(FITC/IgG) was calculated by:

$$F/P = \frac{A_{492\ nm}}{73000} \times \frac{150000}{\text{FITC IgG concentration}} \quad \ldots(8)$$

The F/P ratio as calculated by equation 8 was generally found to lie between 2-3.

The conjugated IgG fractions were aggregated by heating at 63°C for 10 minutes and the aggregates were isolated by gel filtration on a Sephadex G-200 column (2.28 x 80 cm)and similarly aggregates of unconjugated IgG were also prepared.

Freshly isolated lymphocytes were suspended in assay buffer (PBS containing 0.2% bovine serum albumin, 0.02% NaN_3). Three series of tubes were prepared each containing 10^7 cells. In set A, cells were suspended in different concentrations of agg FITC-IgG in PBS containing 1% BSA in the final volume of 1 ml. In set B agg FITC IgG was taken with 100 fold excess of unconjugated agg IgG in order to determine the nonspecific binding. In set C, which served as a standard, duplicates of set A were prepared. The tubes were incubated for two hours with gentle shaking at 4°C. The cells from set A and B were centrifuged at 2000 rpm for 10 minutes and washed twice with cold (8°C) assay buffer. The cells of sets A and B were lysed by resuspending the cell pellet in 2 ml of assay buffer

containing 0.5% NP-40 for 10 minutes at room temperature. The cells from set C were lysed by addition of 1 ml of 1% NP-40 solution. The tubes were again centrifuged for 10 minutes at 2000 rpm and supernatant was taken for fluorescence measurements. Fluorescence was measured on Shimadzu RF 540 spectrofluorophotometer keeping excitation wavelength of 492 nm and emission wavelength of 520 nm.

16. Isolation of lymphocyte membrane IgG binding protein:

IgG binding proteins were isolated either after the solubilization of whole cells or from the isolated membrane (Kulczycki, 1983). Lymphocytes or the membranes were solubilized in PBS containing 0.5% NP-40, 2 mM PMSF, 3 mM EDTA. 10 mM iodoacetamide for 1 hour in cold. Then the mixture was centrifuged at 6000 rpm for 30 minutes and the clear supernatant was first incubated with 8 ml of BSA Sepharose 4B for 2 hours and then with 5 ml of aggregated IgG Sepharose 4B for overnight in cold. The gel was then packed in a syringe (1.4 x 4 cms) and washed with chilled PBS containing 0.1% NP-40. The protein was eluted with 0.5 M acetic acid containing 0.1% NP-40. The fractions (2 ml each) were collected in tubes already containing 0.7 ml of 2 M Tris to neutralize the eluted protein without any delay. The protein was estimated by the method of Cabib and Polacheck (1984).When HPLC of the protein was to be done the protein was eluted with 0.5 M acetic acid devoid of NP-40.

For experiments on rebinding of the protein to affinity gel the eluted protein was first dialysed against 10 mM sodium phosphate buffer pH 7.4 containing 0.05 mM NaCl and 0.1% NP-40 to remove excess of tris used for neutralization. It was then incubated with affinity gel for overnight and washed with PBS. The bound protein was again eluted with 0.5 M acetic acid.

For solubilization of membrane proteins 0.5% NP-40 was used. At this detergent concentration NP-40 effectively solubilized membrane proteins without interfering in their ability to react with specific ligands. The disadvantage of using NP-40 was that it absorbs highly in the ultra violet region of 260-290 nm. Optical density of 0.05% NP-40 at 276 nm was found to be 1.46 and thus NP-40 containing solutions cannot be used for optical measurements in the ultra violet region. Another disadvantage of this detergent is that it interfers strongly in the protein estimation by Lowry's method (Lowry et al., 1951) and dye binding method (Bradford, 1976). Thus the protein in NP-40 containing buffer was estimated by modified Lowry's method (Cabib and Polacheck, 1984). Use of sodium deoxycholate for the solubilization of membrane protein was also tried. It has the advantage over NP-40 that it does not absorb in the ultra-violet region. Optical density of 5% sodium

deoxycholate solution being 0.13 at 280 nm. However sodium deoxycholate precipitates in acidic pH and forms large micelles with increasing concentration of NaCl in the buffer resulting in the formation of highly viscous solution (Tanford and Reynolds, 1976).

To confirm that isolated protein specifically binds to immunoglobulins, the isolated protein was conjugated with FITC by the method of Schreiber and Haimovich (1983) and the conjugate was used to ascertain its binding to the aggregated IgG Sepharose 4B gel. FITC receptor (0.08 mg) was treated with 3 ml of HAIgG Sepharose gel in PBS pH 7.4 containing 0.5% BSA and 0.1% NP-40 in the presence and absence of 9.5 mg of goat IgG in the total volume of 5 ml. The bound protein was eluted with 1 M Tris and fluorescence measured at 520 nm (λ max for excitation 492 nm). The values of fluorescence of eluates in the presence and absence of IgG were F_1 and F_2 respectively. In an additional experiment agg IgG Sepharose was replaced with 5 ml of BSA Sepharose and the fluorescence (F_3) of the eluate recorded similarly. The extent of specific binding of the receptor would be $(F_2-F_3)/F_2 \times 100$. The amount of receptor dissociated in the presence of 9.5 mg IgG will be $(F_2-F_1)/F_2 \times 100$.

17. Affinity chromatography of lymphocyte membrane IgG binding protein on concanavalin A Sepharose 4B gel:

The glycoprotein nature of the IgG binding protein was studied by its ability to specifically interact with

concanavalin A Sepharose 4B gel The protein in TM buffer (10 mM Tris HCl pH 7.5 containing 0.15 M NaCl, 1 mM $CaCl_2$ 1mM $MnCl_2$, 1 mM $MgCl_2$ and 0.1% NP-40) was applied on concanavalin A Sepharose 4B column (1 x 5 cms) equilibrated with the same buffer. The column was operated at a slow rate of 1 ml/hour. It was then washed with TM buffer and bound protein eluted with 0.5 M glucose in TM buffer. The protein fractions were monitored by the method of Cabib and Polacheck (1984).

18. Interaction of cell surface immunoglobulin binding proteins with immunoglobulins and its derivatives :

The receptor was assayed by a method essentially due to Kuriţa et al. (1985). ELISA plates were first incubated with 1% BSA in 0.2 M carbonate buffer pH 9.8 to block all the protein binding sites on the plates. Lymphocytes (0.1 ml containing 10^5 cells) were then added in each well of ELISA plates and left in cold ($8^{\circ}C$) for 2 hours for the cells to settle down. The cells were then fixed with 0.1 ml of 0.5 % glutaraldehyde for 2 hours. (Stocker and Heusser, 1979) and then washed 3 times with 0.3 ml of PBS containing 0.01% NP-40. This was followed by a 2 hour incubation with 0.1 ml of 1% BSA in PBS with 0.1 N glycine to block excess of glutaraldehyde. After three rinses of 0.3 ml each with PBS-NP-40 the cells were incubated for 2 hours with 0.1 ml of either monomeric human IgG,

aggregated human IgG, $F(ab_2')$ (each 0.1 mg/ml in 1% BSA) and in some cases human IgG preincubated with soluble protein A for 1 hour. Unbound IgG was washed with PBS-NP-40. $F(ab_2')$ of antihuman IgG conjugated with peroxidase was first diluted to 400 times with 1% BSA solution in PBS and then 0.1 ml of the resulting mixture was added to the wells and incubated for 2 hours at 30°C. The wells were then washed with PBS-NP-40. After adding 0.2 ml of O.P.D. (0.1%) and H_2O_2(0.05%) in 0.2 M sodium citrate buffer pH 5.0 the reaction was stopped by adding 0.1 ml of 3N HCl and after appropriate dilution with water absorbance was recorded at 492 nm. To check for the nonspecific binding of antihuman IgG peroxidase conjugate, in two wells containing only lymphocytes ,antihuman IgG peroxidase conujugate was added. Substrate was added to find out spontaneous breakdown in two wells containing only lymphocytes. In order to determine the effect of pH and IgG binding to goat lymphocytes human IgG (0.1 mg / ml in 1% BSA) was taken in 0.06 M sodium citrate buffer in the pH range of 3 to 5 and in 0.06 M sodium phosphate buffer in the pH range of 6to8..The effect of ionic strength on the binding of IgG to cell surface was studied in 10 mM phosphate buffer containing 0.1 M to 0.8 M NaCl. Rest of the procedure was same.

III RESULTS

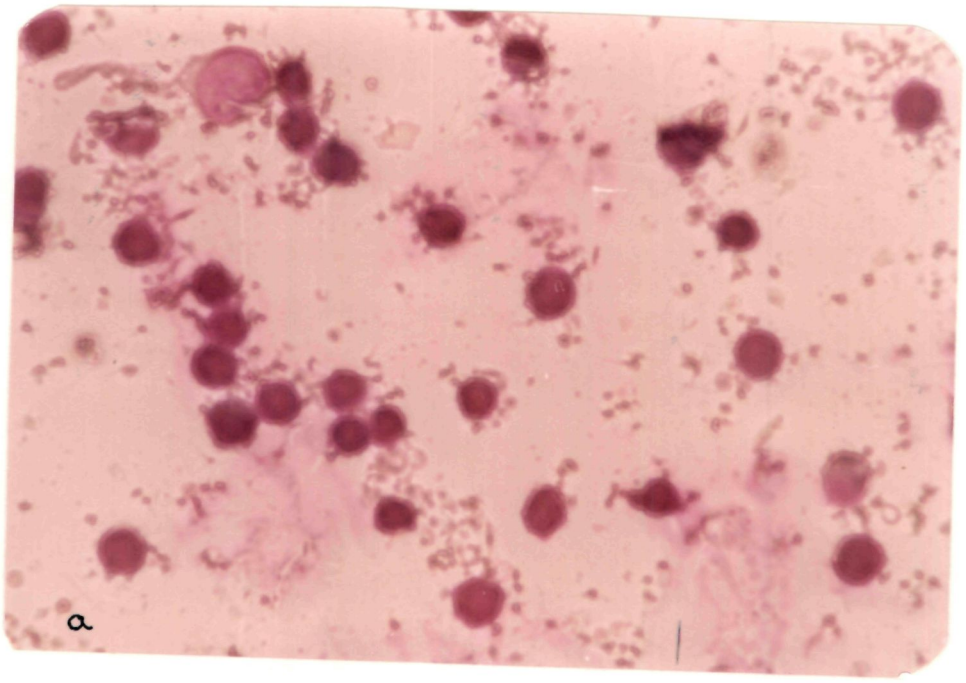
1. Isolation of lymphocytes from goat blood:

Lymphocytes were isolated from fresh goat blood and stored in 0.72% sodium citrate solution. On differential leukocyte count by Giemsa staining the goat blood was found to contain 74% lymphocytes, 21% neutrophiles and 5% monocytes. No eosinophils were detected. From 1.5 litres of goat blood about 60 ml of buffy coat was obtained.Lymphocytes were isolated by density gradient centrifugation of buffy coat on Histopaque. Contaminating RBCs were lysed by 0.83% NH_4Cl and then the cells were washed with normal saline. About 10^8 cells were isolated. A thin smear of cell suspension was made on a glass slide and stained with Giemsa. Apart from normal lymphocytes, some other cells with large opened up chromatin in the nucleus and scant cytoplasm were also seen (see Figure 13). On the marphological basis these cells appear to be large lymphocytes or blasts. On the basis of Giemsa staining the suspension was found to contain 96% lymphocytes and 4% neutrophils. The viability of the lymphocytes was ascertained by trypan blue exclusion method (Mishell and Shiigi, 1980). Cell viability was generally 90-93%.

Figure 13

Photograph of isolated goat peripheral blood lymphocytes.

Lymphocytes were isolated and stained with Geimsa stain as described in the experimental section . The photographs were taken on Nikon Optiphot microscope with UFX photographer attachement using a diffusion filter. Magnification(a) 50 x (b) 500 x. Enlargement during photoprinting has not been considered.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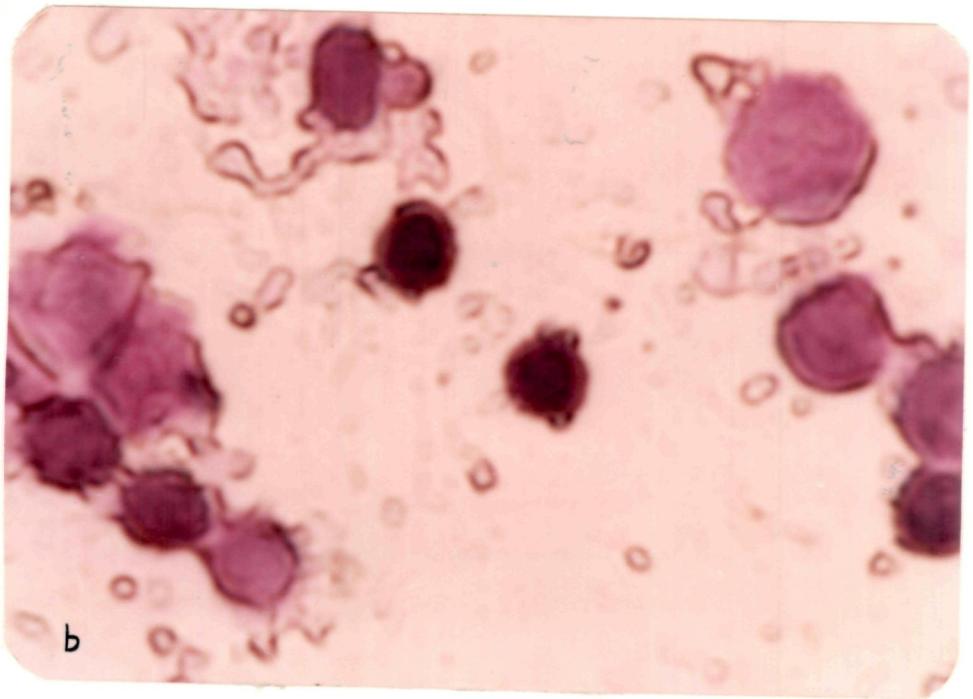
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2. Detection of IgG binding protein on lymphocyte surface:

The presence of IgG binding protein on the surface of lymphocytes was detected by studying the binding of FITC conjugate of aggregated IgG (agg FITC-IgG)to goat lymphocytes. The binding of heat aggregated IgG to IgG receptor on lymphocytes was studied by incubating 4.5 x 10^7 cells in the binding buffer (10mM sodium phosphate buffer, pH 7.4 containing 1% BSA and 0.2% NaN_3) with increasing concentration (2-9 µg/ml) of agg-FITC-IgG at 8^oC for 2 hours. The amount of agg-FITC-IgG bound was determined spectrofluorometerically at 520 nm using an excitation wavelength of 492 nm. The results are graphically shown in Figure 14. It can be seen in Figure 14 that the binding of IgG increased substantially with increase in IgG concentration upto 7.0 µg/ml beyond which further increase in the IgG concentration caused very little change in the IgG binding. The curve seems to slope off at a conc of 0.7 µg/ml which will be equivalent to 4.6 x 10^{-9} M of IgG monomers. This would mean that about $6x10^7$ IgG monomer molecules were bound per cell. It should be pointed out that the maximum error in these experiments was about 4% which would mean that the number of IgG molecules bound per cell is $6x10^7 \pm 2.4x10^6$. Assuming an stoichiometery of IgG to receptor of 1:1 these

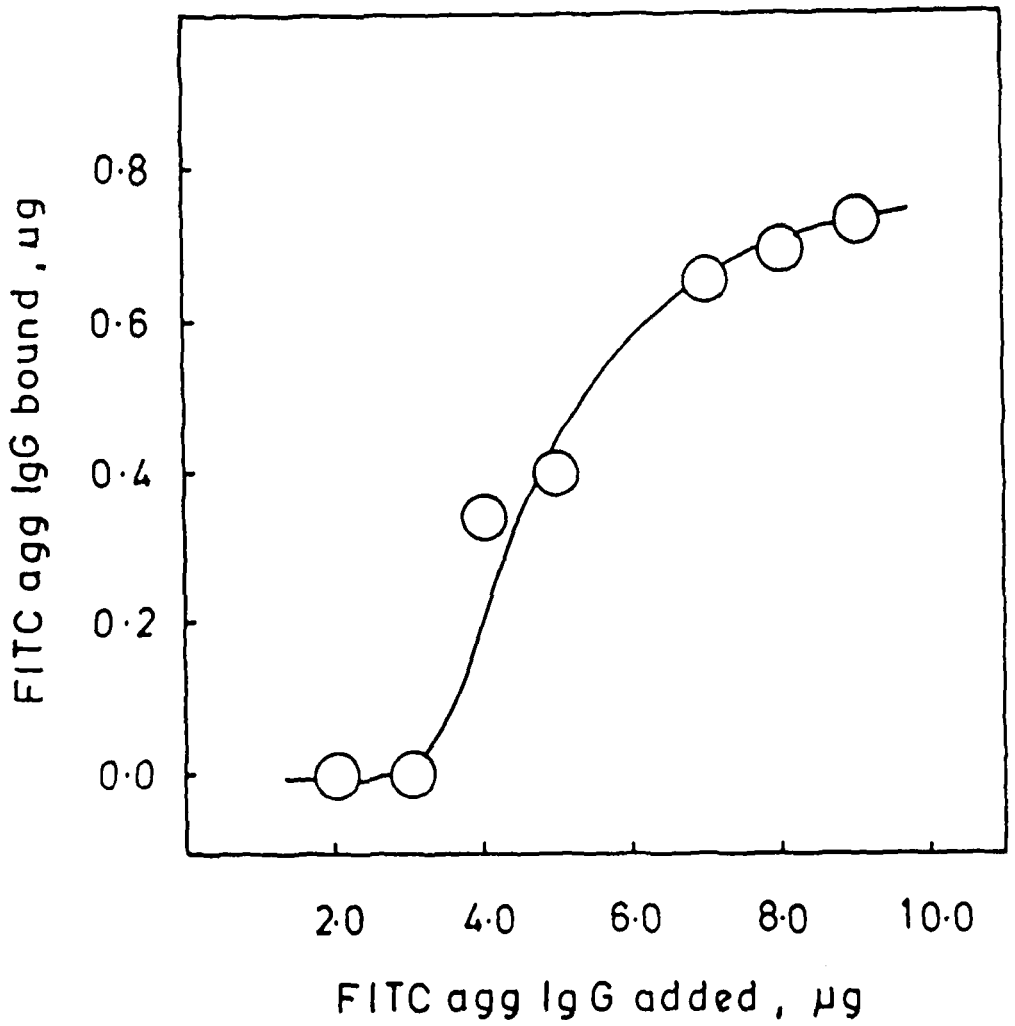


Figure 14.

Detection of IgG binding protiens on the surface of goat peripheral blood lymphocyte by specific binding of fluorescein conjugated heat aggregated goat IgG.

Aggregated-FITC-IgG_7 (2-9 μg) was incubated at $8^{o}C$ for 2 hours with 4.5 $x10^{7}$ lymphocytes. The bound agg-FITC-IgG was detrmined spectrofluorometrically as described in methods. Values are average of two experiments.

results would indicate the presence of about 10^7 moles of receptor on the cell surface.

3. Isolation of lymphocyte membrane IgG binding protein:

IgG binding protein from goat peripheral blood lymphocytes was isolated both from the cell homogenates as well as from isolated cell membrane by affinity chromatography on heat aggregated IgG Sepharose 4B column.

(a) Isolation of IgG binding protein from whole cells

From $9x10^8$ lymphocytes about 61 mg of protein was solubilized in 10 mM sodium phosphate buffer pH 7.4 containing 0.15 M NaCl, 0.5% NP-40, 2 mM PMSF, 3 mM EDTA and 10 mM iodoacetamide. The solubilized protein was subjected to affinity chromatography on heat aggregated IgG Sepharose 4B column (1.4x5 cm) and the amount of protein eluted with 0.5 M acetic acid from the column was 0.68 mg. The elution profile is shown in Figure 15 . On rechromatography on the affinity column, the protein eluted with identical elution volume (9 ml) as can be seen in Figure 16. The amount of IgG binding protein then obtained was 0.45 mg. Thus the final protein yield was less than 1%.

(b) Isolation of IgG binding protein from isolated membrane

IgG binding protein were also isolated from crude

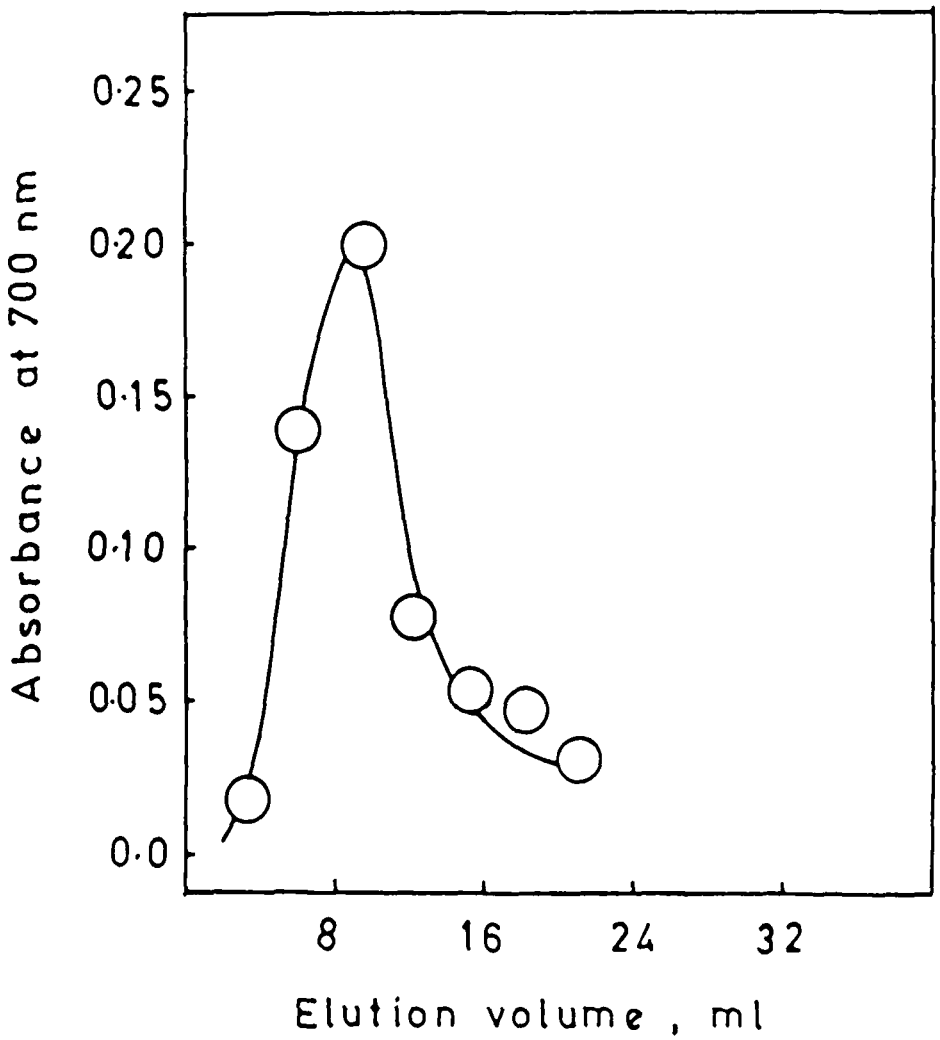


Figure 15.

Affinity chromatography of protein solubilized from whole cells by NP-40 on aggregated IgG Sepharose 4B gel.

About 61 mg of protein was incubated with 8 ml of aggregated IgG Sepharose 4b gel for overnight. After packing the gel in syringe (1.4 x 5 cms) the unbound protein was washed with PBS containing 0.1%. NP-40. The bound protein was eluted with 0.5 N acetic acid containing 0.1% NP-40 in 3 ml fractions. The column was monitored by the method of Cabib and Polacheck(1984).

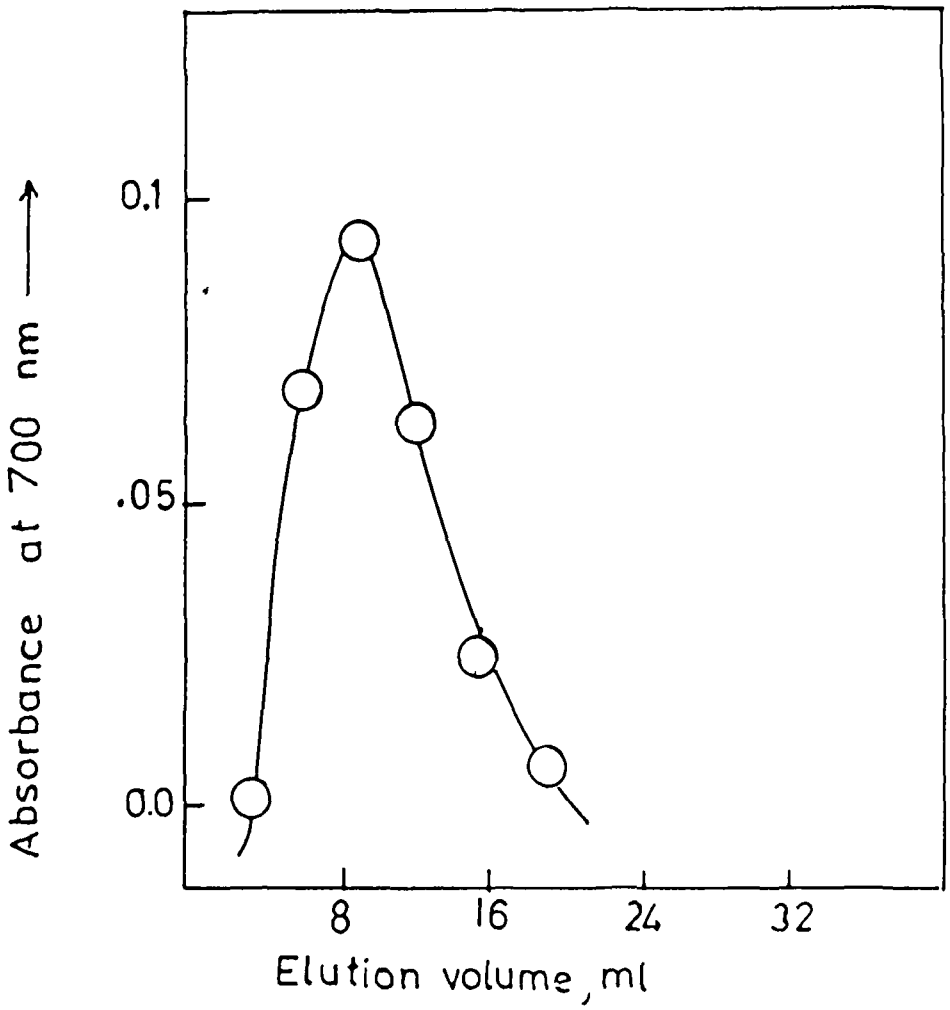


Figure 16.

Rebinding of IgG binding protein isolated by affinity chromtography to aggregated IgG Sepharose 4B gel.

About 0.5 mg of protein was applied on aggregated IgG Sepharose 4B column (1.4 x 5 cms). After washing, the bound protein was eluted with 0.5 N acetic acid. The column was monitored by the method of Cabib and Polacheck (1984).

lymphocytes membrane preparations . Lymphocytes were lysed by hypotonic lysis and then membrane fraction was prepared by the method of Coligan and Kindt (1986). About $3x10^9$ cells were lysed and homogenised. The homogenate was found to contain 88.5 mg of protein. The crude membrane fraction was solubilized in 10 mM sodium phosphate buffer, pH 7.4 containing 0.15 M NaCl, 0.5% NP-40, 2 mM PMSF, 3 mM EDTA and 10 mM iodoacetamide and was found to contain 6.0 mg of protein. The membrane fraction was assayed by measuring 5' nucleotidase activity as recommended by Jakoly and Morre (1971). Specific activity of enzyme in terms of inorganic phosphate released was found to be 0.33 μg Pi/mg/min and 1.27 μg Pi/mg/min for cell homogenate and soluble membrane fraction respectively. In other experiments also the 5' nucleotidase activity of the membrane was found to be significantly higher. About 6.0 mg of membrane protein was incubated with 6 ml of aggregated IgG Sepharose 4B gel equilibrated with 10 mM sodium phosphate buffer pH 7.4 containing 0.15 M NaCl and 0.1% NP-40. The elution profile is depicted in Figure 17. The amount of protein eluted from the column with 0.5M acetic acid was 0.16 mg. Thus the final protein yield was 0.2%. Similar observations were made by Takacs who obtained 0.5-0.8% of solubilized membrane material of human peripheral blood lymphocytes on affinity chromatography on IgG Sepharose column: the bound

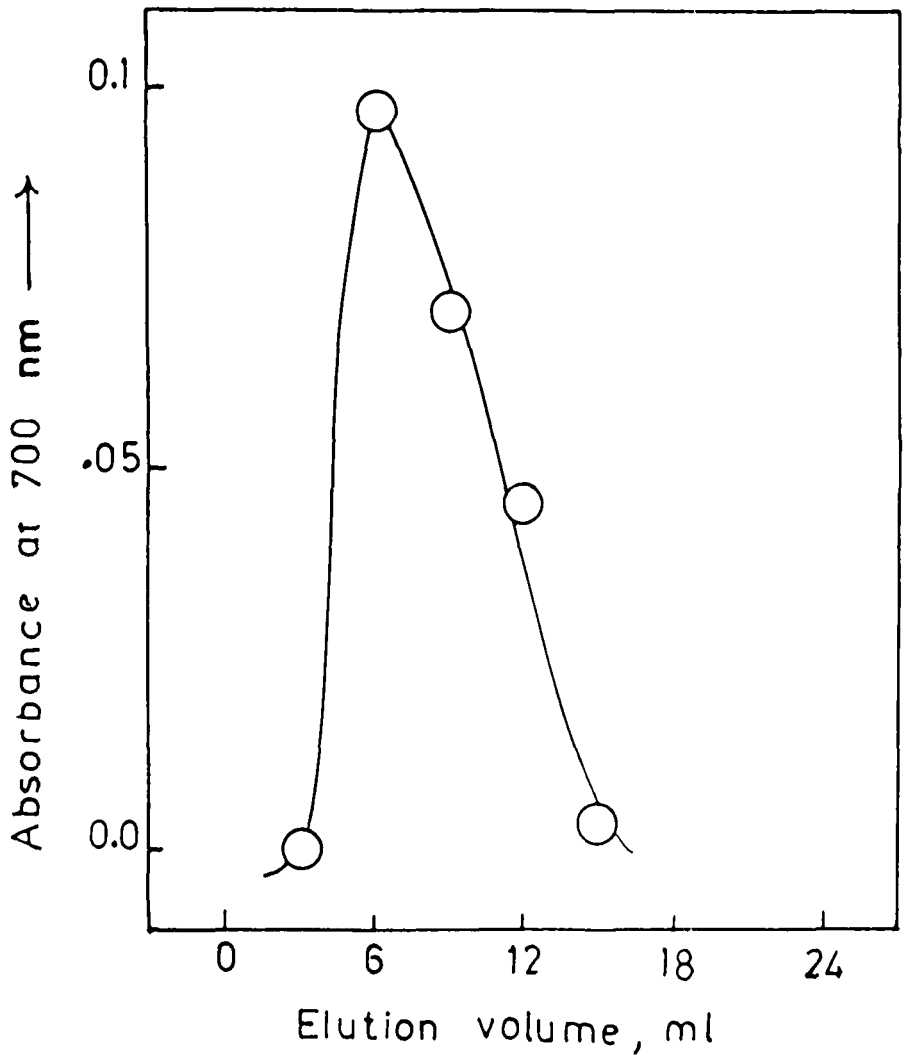


Figure 17.

Affinity chromatography of NP-40 solubilized lymophocyte membrane on aggregated IgG Sepharose-4B gel.

About 6 mg of protein was incubated with 8 ml of aggregated IgG Sepharose 4B gel for overnight. After packing the gel in syringes (1.4 x 5 cms) the unbound protein was washed with PBS. The bound protein was eluted with 0.5 M acetic acid in 3 ml fractions. The column was monitored by the method of Cabib and Polachek (1984)

protein was eluted with 0.2M glycine HCl pH 2.5, containing 0.5 M NaCl, 0.5% NP-40

4. Rebinding of FITC conjugated IgG binding protein to the affinity gel

In order to see that the isolated IgG binding protein is active and specifically binds IgG, rebinding of FITC conjugated protein to the affinity gel in the presence and absence of soluble IgG was studied. About 80 μg of FITC conjugated IgG binding protein was first incubated with 9.5 mg of agg IgG for 2 hours at 8^{o}C and then incubated with 3 ml of agg IgG Sepharose 4 B gel for 6 hours, the final volume being 5 ml. Alternatively the FITC conjugated receptor was bound to BSA Sepharose 4B gel also. The bound protein was eluted with 1 M Tris and measured spectrofluorometerically at 520 nm with an excitation wavelength of 492 nm. The results are shown in Table VII. It was found that incubation of IgG binding protein with aggregated IgG Sepharose 4B gel in the presence of aggregated IgG results in 70-75% inhibition of binding. This shows that the eluted protein was active and specific for IgG. By comparing the amount of protein bound to aggregated IgG Sepharose 4B gel, it was found that 63-69% of the protein specifically rebinds affinity gel.

TABLE VII

Rebinding of FITC labelled lymphocyte membrane IgG binding protein to agg IgG Sepharose 4B gel

Exp. no.	Pre-incubation	Protein Sep. Column	Relative Fluorescence of eluent	% inhibition[a]	% specific binding[b]
1. F_3	Buffer[c]	BSA Sep	29.2		
F_2	Buffer	Agg IgG Sep	95.0		
F_1	IgG	Agg IgG Sep	28.5	70%	69%
2. F_3	Buffer	BSA Sep	22.9		
F_2	Buffer	Agg IgG Sep	82.1		
F_1	IgG	Agg IgG Sep	20.0	75.6%	72%
3. F_3	Buffer	BSA Sep	32.8		
F_2	Buffer	Agg IgG Sep	90.1		
F_1	IgG	Agg IgG Sep	25.4	71.8%	63.5%

(a) $F_2-F_1 / F_2 \times 100$
(b) $F_2-F_3 / F_2 \times 100$
(c) 10 mM phosphate buffer pH 7.4 containing 0.15 M NaCl.

5. Binding of immunoglobulins and its derivatives to intact lymphocytes:

Binding of human IgG monomer, aggregated IgG and $F(ab_2')$ fragment to peripheral goat blood lymphocytes was studied by ELISA using peroxidase conjugated $F(ab_2')$ of anti-human IgG under different experimental conditions of pH and ionic strength.

(a) Effect of pH:

In order to determine the binding of IgG and its derivatives by specific cell surface lymphocyte receptors, IgG and its derivatives were exposed to desired acid or alkaline pH and the cells were treated similarly. The cells were then allowed to react with its ligand for 2 hours at 8°C. The complex was then treated with peroxidase conjugated $F(ab_2')$ fragment of anti-human IgG for 2 hours at 8°C in 10 mM sodium phosphate buffer pH 7.4, containing 0.15 M NaCl and the peroxidase activity measured at 37°C as described in the experimental section. The peroxidase activity provided a measure of binding of IgG and its derivative by the lymphocytes. The results obtained at various pH with 10^5 cells are depicted in Figure 18 where it can be seen that the binding of IgG monomer to the cells is not markedly altered by change in pH from 3-8. However the binding of heat aggregated IgG showed pronounced dependence on pH. The binding of $F(ab_2')$ to

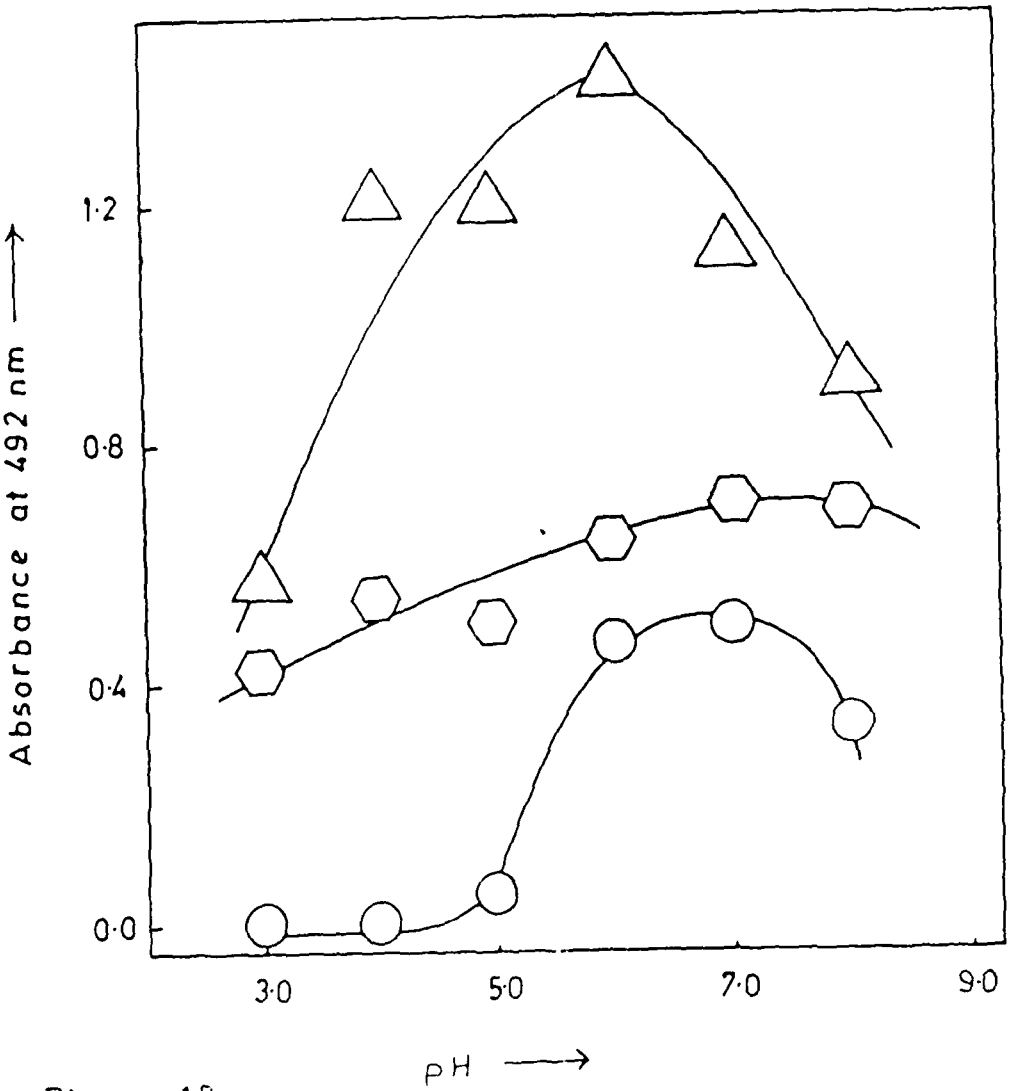


Figure 18.

Effect of pH on the interaction of IgG binding proteins on goat lymphocyte surface with immunoglobulin and its derivative.

Ten µg of aggregated IgG (Δ) monomeric IgG (⬡) and its F(ab_2') fragment (O) were incubated with 10^5 cells in 60 mM sodium phosphate buffer (pH 6-8) or 60 mM sodium acetate buffer (pH 3-5) for 2 h and the bound protein measured by ELISA using peroxidase conjugated F(ab_2') of antihuman IgG as described in the experimental section. Results are average of three experiments.

cells seem to be weak but significant. The maximum binding occurred at pH 6-7. Strikingly, no binding of $F(ab_2')$ to cells occurred below pH 5.0. In contrast, the binding of heat aggregated IgG to cells was substantial near pH 4-5. The maximum binding of heat aggregated IgG to cells occurred near pH 6.0. Another important feature is that the IgG receptor retains significant activity even at pH 3.0.

(b) Effect of ionic strength:

The effect of ionic strength on the binding of immunoglobulins and its derivatives namely aggregated IgG and $F(ab_2')$ was investigated in 10 mM sodium phosphate buffer, pH 7.0 (ionic strength 0.02) containing different concentration of NaCl (0-0.8M). The cell and the ligands were exposed to the desired ionic strength for 2 hours at $4^{o}C$ and the binding measured by peroxidase conjugated $F(ab_2')$ of antihuman IgG in the manner described above. The results are shown in Figure 19. The binding of the ligand appeared to decrease gradually upon increase in ionic strength. This decrease was more pronounced for aggregated IgG.

(c) Effect of Protein A:

To study the effect of protein A on the binding of IgG and its derivatives to goat peripheral blood

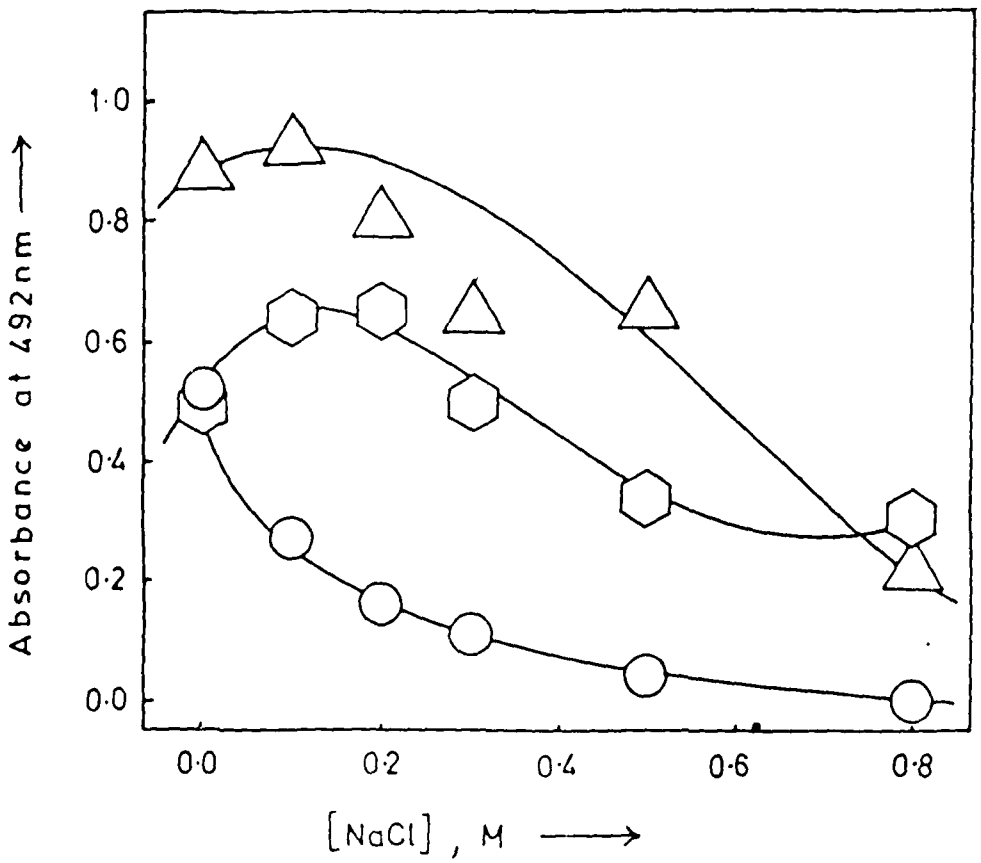


Figure 19.

Effect of ionic strength on the interaction of IgG binding proteins on lymphocyte surface with immunoglobulin and its derivatives.

Ten µg of aggregated IgG (Δ) monomeric IgG ($_5$⬡) and its F(ab_2') fragment (○) were incubated with 10^5 cells in 10 mM sodium phosphate buffer containing 0.0-0.8 M NaCl for 2 h and the bound protein measured by ELISA using peroxidase conjugated F(ab_2') of antihuman IgG as described in the experimental section. Results are average of three experiments.

lymphocytes, 0.1 mg of aggregated IgG and 0.1 mg of monomeric IgG were incubated with 1 mg of protein A. Ten µg of such protein A treated and untreated immunoglobulin were incubated with 10^5 cells in 10 mM sodium phosphate buffer pH 7.4 for 2 hours and the bound protein was measured by peroxidase conjugate of anti-human IgG in the manner described above. The results are depicted in Figure 20 . As can be seen there was about 60% decrease in the binding of monomeric IgG after protein A incubation. This indicate that the immunoglobulins were binding through the Fc region. The fact that the binding of monomeric IgG to lymphocytes was not completely abolished by protein A preincubation but was similar to the extent of binding of $F(ab_2')$ fragments indicates that some IgG is bound through $F(ab_2')$ region also. The binding of aggregated IgG to goat peripheral blood lymphocytes remained almost unaffected by protein A presumably because of the inaccessibility of the latter to the C_H2-C_H3 domains which appear to be involved in the binding of protein A (Biguzzi,1982).

6. Determination of molecular weight of lymphocyte membrane IgG binding protein by SDS-PAGE:

The molecular weight of IgG binding protein was determined by SDS polyacrylamide gel electrophoresis in Tris glycine buffer (25 mM Tris and 194 mM glycine), pH

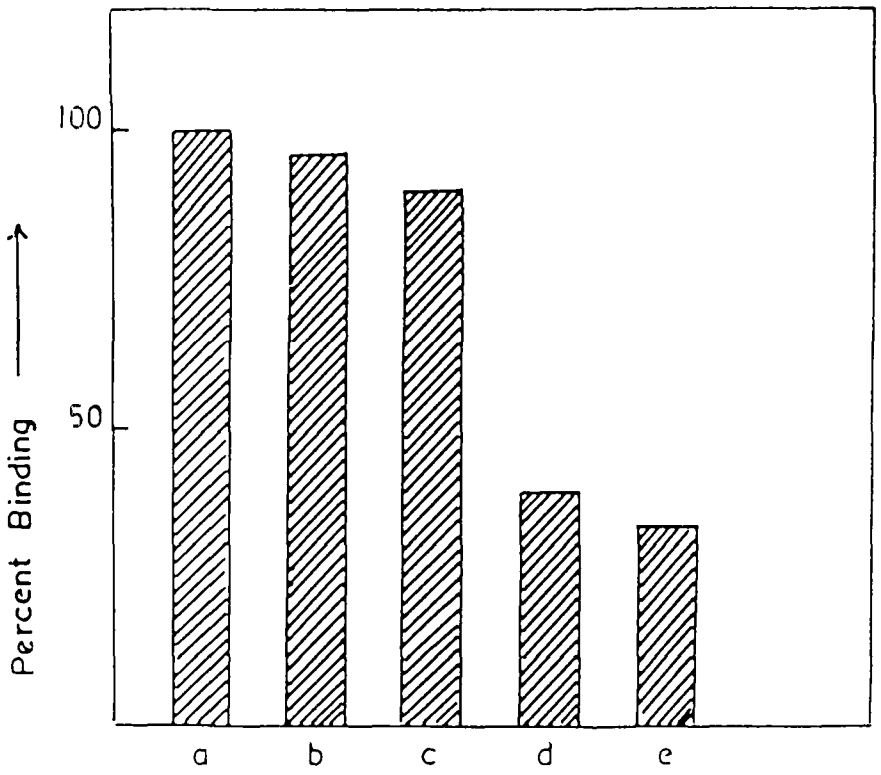


Figure 20.

Effect of protein A on the binding of immunoglobulins and its derivatives to lymphocyte surface.

Aggregated IgG (0.1 mg), and monomeric IgG (0.1 mg) were preincubated with 1 mg of soluble protein A. Ten μg of such immunoglobulins, F(ab_2') and BSA were incubated with 10^5 cells fixed in wells of flat Bottom ELISA plates in 10 mM sodium phosphate buffer pH 7.4 for 2 hours and the bound protein was detected by adding 0.1 ml of 1:400 diluted F(ab_2') of antihuman IgG conjugated to peroxidase. The enzyme activity against O.P.D. was measured spectrophotometerically at 492 nm. (a) agg IgG, (b) agg IgG treated with Protein A, (c) monomeric IgG, (d) monomeric IgG treated with Protein A, (e) F(ab_2'). Results are average of three experiments.

8.3 containing 0.1% SDS according to the method of Laemmli (1970).

The marker proteins namely bovine serum albumin, ovalbumin, IgG (heavy and light chain), cytochrome c and IgG binding protein were electrophoresed under identical conditions. In each case the mobility of the protein band relative to the mobility of the dye i.e. Rm, was determined with an uncertainity of less than 5%. The results are given in Table VIII and are graphically depicted in Figure 22 in the form of a plot of Rm versus log M. The linear plot (see Figure 22) was drawn by the method of least squares and fits the equation

$$\text{Log } M = -1.28\ Rm + 5.026 \qquad \ldots\ (9)$$

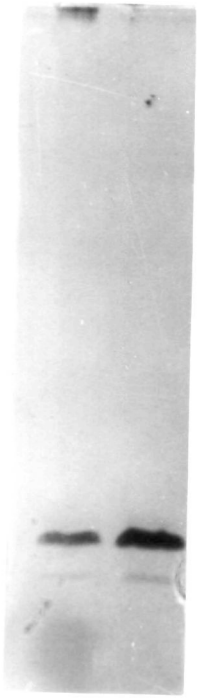
Electrophoretogram of the IgG binding protein isolated from membrane is shown in Figure 21.

Evidently , the IgG binding protein moved essentially as a single band both in the presence and absence of 0.2 M 2-mercaptoethanol with an Rm of 0.69 which according to equation 9 is consistent with Mr of 14000. A faint band which is hardly visible in Figure 21 had an Rm of 0.76 which corresponds to a molecular weight of 11,300. On SDS PAGE the molecular weight of the IgG binding protein which was isolated from whole cells by affinity chromatography was also found to be the same.

Figure 21.

SDS polyacrylamide gel electrophoresis pattern of lymphocyte membrane IgG binding protein.

About 1.5 µg of protein was electrophoresed in 12.5% polyacrylamide slab gel in tris glycine buffer pH 8.3 (25 mM tris and 0.194 M glycine) containing 0.1% SDS for about 3 hours using a current of 50 m A per slab (17 x 20 x 0 2 cm). The gel was stained with silver by the method of Oaklay et al (1980).(a) non reduced (b) reduced



a b

TABLE VIII

Molecular weight and relative mobility for marker proteins used in sodium dodecyl sulphate polyacrylamide gel electrophoresis

Proteins	Molecular weight	log M	Relative Mobility Rm
Bovine serum[a] albumin	68000	4.83	0.16
Ovalbumin[a]	43000	4.63	0.30
Human IgG[b] heavy Chain	51000	4.71	0.23
Human IgG[b] light chain	25000	4.40	0.52
Cytochrome c[a]	11700	4.07	0.74
IgG binding protein			0.69

(a) Hames,1986
(b) Roitt, 1988

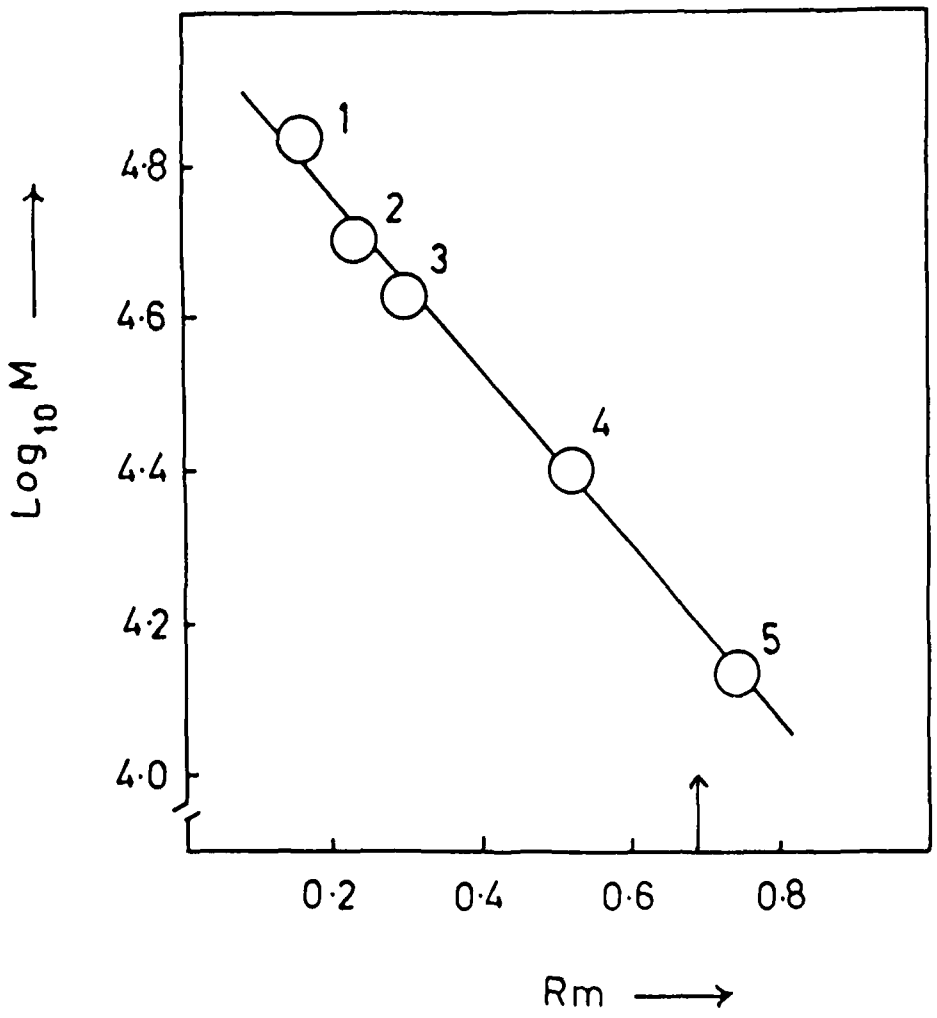


Figure 22.

Plot of Rm values of marker proteins versus logarithm of molecular weight.

The marker proteins were (1) bovine serum albumin (68000) (2) heavy chain of globulin (5$\frac{1}{2}$,000) (3) ovalbumin (43000) (4) light chain of globulin (25000) (5) cytochrome c (1$\frac{1}{2}$700). The Rm of immunoglobulin binding protein is indicated by an arrow. The marker proteins were elctrophoresed under denaturing conditions as described in legend to Fig 21.

7. Glycoprotein nature of lymphocyte membrane IgG binding protein:

The IgG binding protein isolated from lymphocytes was found to beaglycoprotein. Its neutral hexose contents as determined by phenol sulphuric acid method (Dubois et al., 1956) was 11 ± 1% : the uncertainty in the carbohydrate estimation being about 9%. The number of hexose per 14000 g of the IgG receptor thus comes out to be 8.56 ± 0.78. The nature of carbohydrate moiety of the IgG binding protein was determined by its reactivity towards concanavalin A Sepharose 4B column equilibrated in TM buffer ie. 10 mM tris HCl, pH 7.5 containing 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$ and 0.1% NP-40. The IgG binding protein was specifically eluted with 0.5 M glucose in TM buffer . The behaviour of the IgG receptor from lymphocyte membrane was also found to be similar. Since concanavalin A specifically binds mannose/glucose or there appropriate derivatives (Leiner et al, 1986)the results of Figure 23. suggest the presence of mannose/glucose or there derivatives in carbohydrate moeities of the IgG receptor. The IgG binding protein was devoid of sialic acid.

8. Optical properties of lymphocyte membrane IgG binding proteins:

The light absorption spectra of the IgG binding protein from lymphocytes was studied in the UV region,

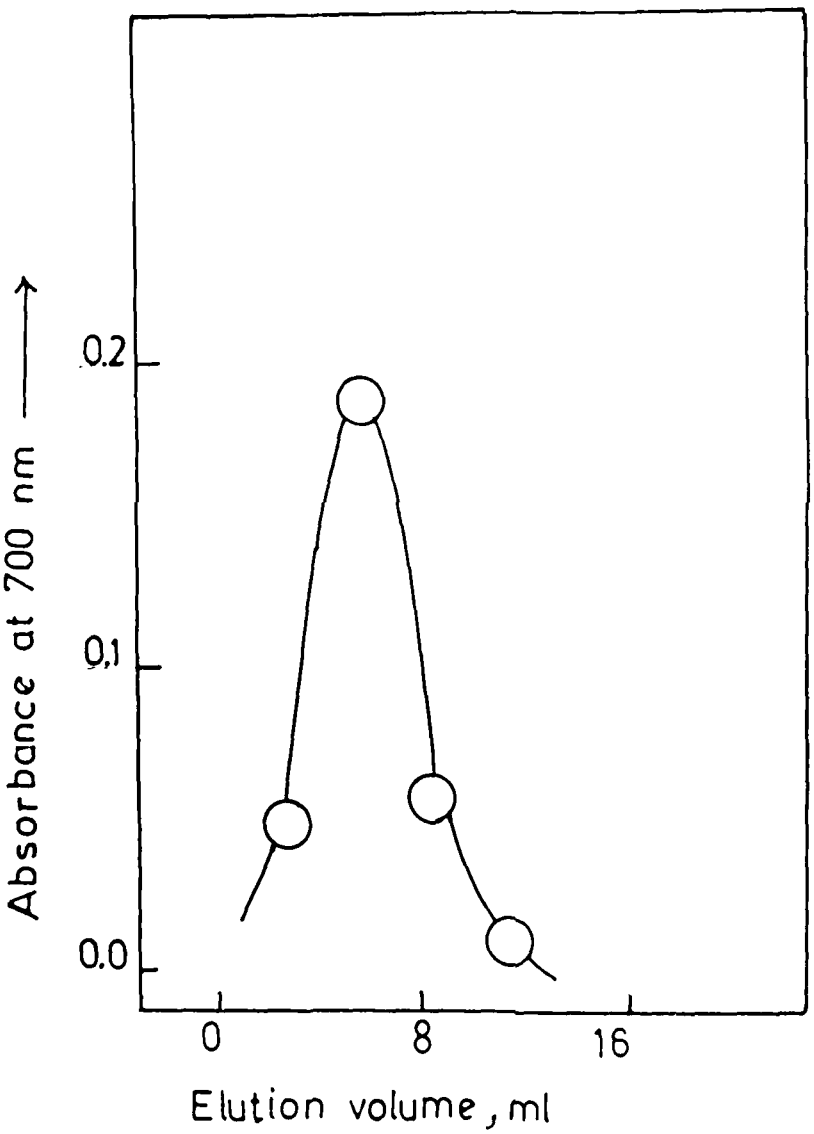


Figure 23.

Affinity chromatography of membrane IgG binding protein on concanavalin A Sepharose 4B gel.

About 489 µg of lymphocyte membrane IgG binding protein was applied on concanavalin A Sepharose 4B gel (1x5 cm) equilibrated in TM buffer. (10 mM Tris HCl pH 7-5 containing 0.15 M NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$ 1 mM $MgCl_2$ and 0.1% NP-40.

wavelength 230-350 nm in 10 mM sodium phosphate buffer pH 7.4 containing 0.15 M NaCl and 0.1% sodium deoxycholate. The results are graphically shown in Figure 24. It can be seen in the figure that the maximum absorption occurred near 278 nm. The fluorescence excitation and emission spectra of the same solution of IgG binding protein was studied in the wavelength regions of 200-300 nm and 300-400 nm in 10 mM sodium phosphate buffer pH 7.4 containing 0.1%deoxycholate, respectively. The results are shown in Figure 25 where it can be seen that the excitation maxima and emission maxima occur, respectively at 277.6 nm and 341.4 nm. The fluorescence excitation and emission spectra of model compound such as N acetyl-tryptophanamide, N acetyl-L-tyrosine ethyl ester and N acetyl-L-phenylalanine ethyl ester were also measured under identical conditions(see Figure 26). The excitation and emission maxima were located and are listed in Table IX .The results of table shows that the aromatic chromophores present in the IgG binding protein include among others, tryptophan residues.

9. Gel filtration behaviour of lymphocyte membrane IgG binding protein :

The IgG binding protein isolated from lymphocytes was subjected to gel filtration on HPLC Shim Pack Diol 150 column (0.785 x 25 cm) in 10 mM scdium phosphate buffer pH

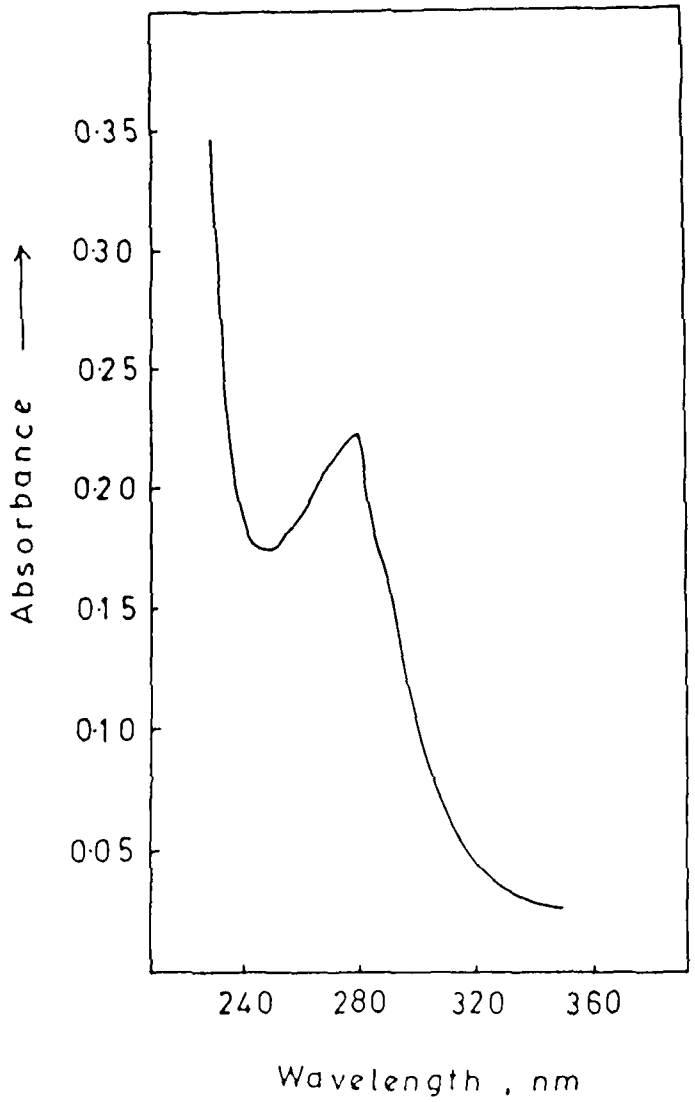


Figure 24.

Ultraviolet absorption spectra of lymphocyte membrane receptor for IgG binding protein in aqueous buffer.

The protein solution (0.08 mg/ml) was taken in 1Ø mM sodium phosphate buffer pH 7.4 containing 0.15 M NaCl and 0.1% sodium deoxycholate. The absorbance was measured at each wavelength on Cecil double beam spectrophotometer, model CE 594.

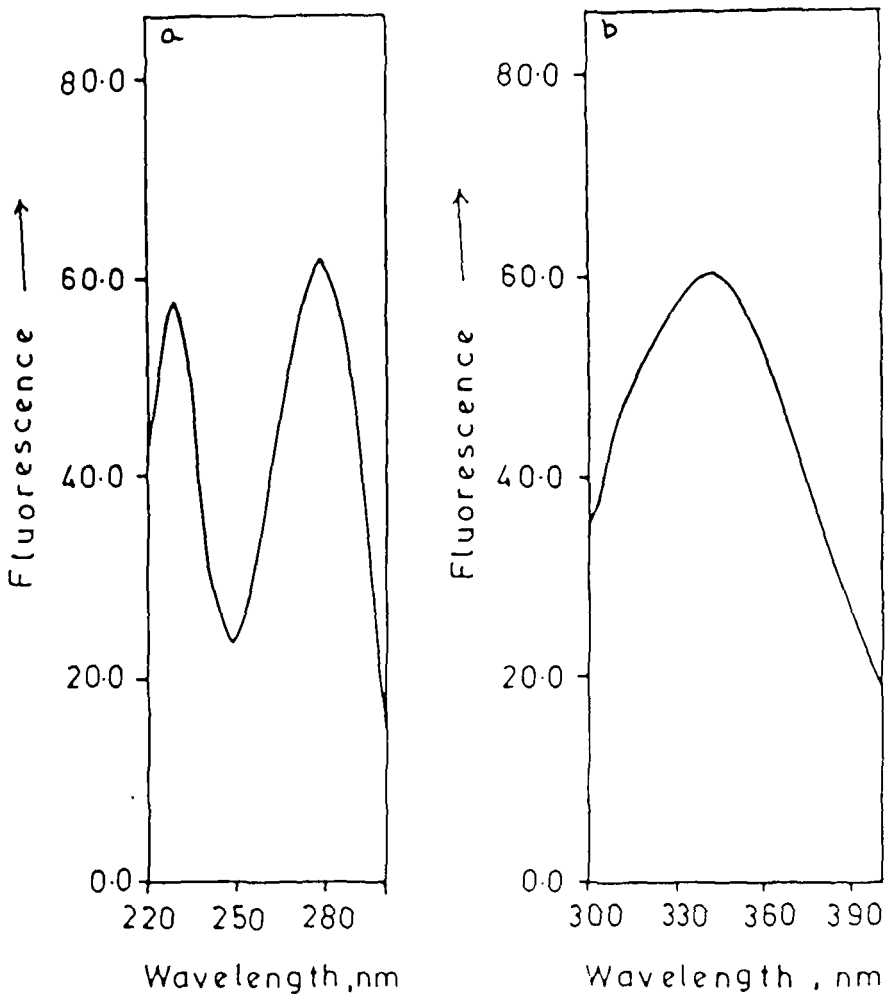


Figure 25.

Excitation (a) and emission (b) spectra of membrane IgG binding protein in aqueous buffer.

The protein solution in 10 mM sodium phosphate buffer pH 7.4 containing 0.15 NaCl and 0.1% sodium deoxycholate contained 0.0 8 mg/ml of protein. The spectra was recorded at room temperature (26^{o}C) using 10 nm slit.

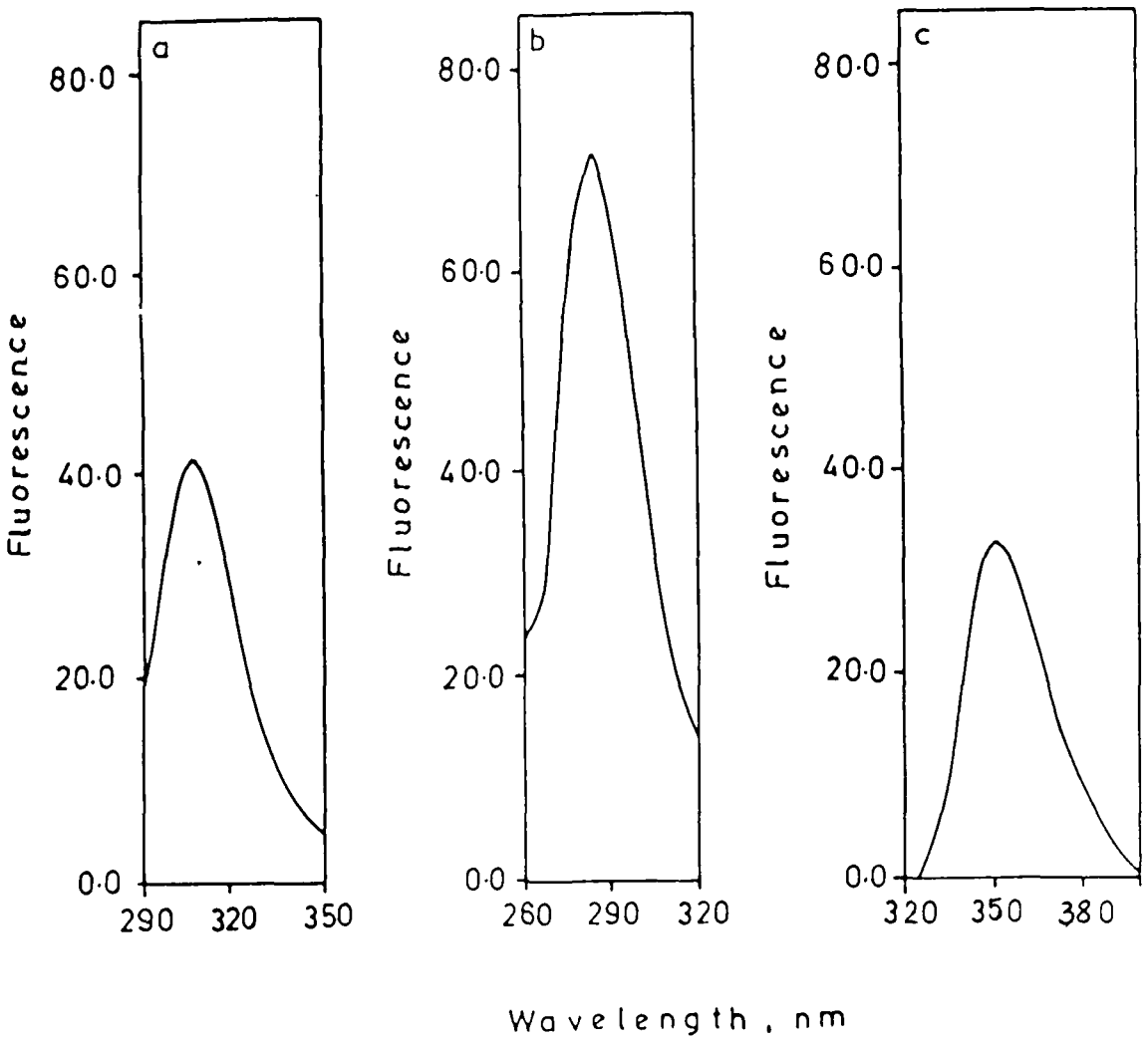


Figure 26.

Emission spectra of derivatives of aromatic amino acids. Amino acids (0.02-0.04 mg/ml were taken in 10 mM sodium phosphate buffer pH 7.4 containing 0.15 M NaCl and 0.1% sodium dexoxycholate. the spectra was recorded at room temperature (26°C) using 10 nm slit. The excitation wavelengths were 275.5, 262.2 and 288.7 nm for (a) N acetyl-tyrosine-ethylester (b) N acetyl-phenylalanine-ethyl ester and (c) N acetyl-L-tryptophanamide respectively.

TABLE IX

The excitation and emmission maxima for IgG binding protein and model compounds

Substance	Excitation max nm	Emission max nm
1. N acetyl-L-tryptophana mide, 0.02 mg/ml	288.7	355.8
2. N acetyl-L-Tryosine ethyl ester, 0.02 mg/ml	275.2	306.2
3. N acetyl-L-Phenylalanine ethyl ester, 0.04 mg/ml	262.2	284.9
4. IgG binding protein	277.6	341.4

7.4 containing 0.5 M sodium chloride and 0.02% sodium azide. It can be seen in Figure 27 that a well defined protein peak was eluted with a elution vol of 6.46 (see Figure 27,peak C). The shoulder A and the peak B were eluted with average retention time of 5.29 min and 5.77 min (see Figure 27, Peak A, B). The relative concentration of the protein under peaks A, B and C were 18, 12 and 60 per cent , respectively. The gel filtration results are summarized in Table.X The results were normalized in terms of function $F(\nu)$ according to the equation,

$$F(\nu) = (V_e^{1/3} - V_t^{1/3}) / (V_o^{1/3} - V_t^{1/3}) \quad ...(10)$$

given by Squire (1985) where V_t is the total volume of the column, V_e is the elution volume and V_o is the void volume of the column. The total volume of the column V_t was calculated from its diameter (0.785) and height (25 cm) and the value was 12.25 ml. In order to find out the void volume V_o of the column heat aggregated IgG was passed through the column. Its elution volume was 4.82 ml. The retention time listed in column 2 of Table X was obtained at a flow rate of 1 ml/min and therefore, represents the elution volume V_e of the protein. The marker proteins listed in Table XI were used for the calibration of the column. The value of $F(\nu)$ calculated by the help of above equation are listed in Table XI. A curve was obtained between $F(\nu)$ and $M^{1/3}$ for the marker

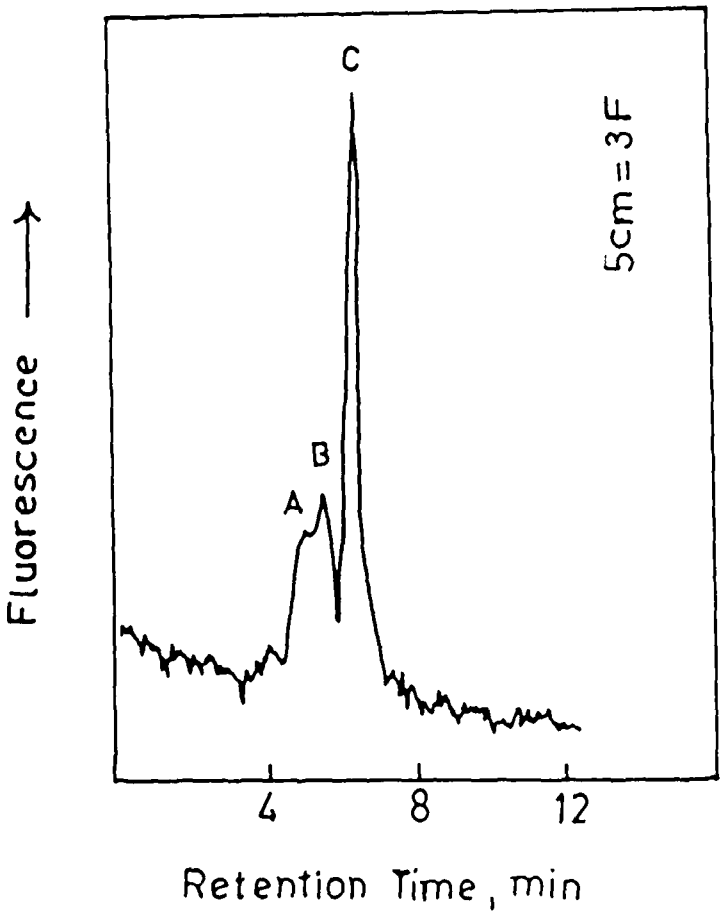


Figure 27.

The HPLC gel filtration of lymphocyte membrane IgG binding protein.

About 1.2 µg of IgG binding protein in 10 mM sodium phosphate buffer pH 7.4 containing 0.5 M NaCl was gel filtred on HPLC Shim Pack Diol 150 column (.79 x 25 cm) equilibrated in the same buffer operating at a flow rate of 1 ml/min maintained with Shimadzu LC-6A pump. The protein peaks were detected spectrofluorometerically at 354 nm keeping excitation at 280 nm. The slit used for both the beams was 15 nm. The peaks were recorded automatically by C-R3A integrator.

TABLE X

Gel filtration of lymphocyte membrane IgG binding protein on Shim Pack Diol 150 column[a]

Peak	Retention Time, min.	Relative conc.	F(ν)	Molecular wt.	Stokes Radius (nm.)
A	5.29	18%	0.913	119000	4.54
B	5.77	22%	0.829	94000	4.07
C	6.46	60%	0.717	67000	3.48

a, Details of peak shown in figure 27

TABLE XI

Gel filtration of marker proteins for calibration of Shim Pack Diol -150 column.

Marker Proteins[a]	Molecular weight (M)	$M^{1/3}$	Stokes Radius nm	Retention time min.	$F(\nu)$
1. Bovine Serum Albumin	69000	41.057	3.50	6.51	0.711
2. Ovalbumin	43000	35.034	3.00	7.07	0.627
3. Chymotrypsinogen A	25700	29.510	1.94	8.40	0.442
4. Trypsinogen	24000	28.845	1.90	8.35	0.448
5. Ribonuclease A	13700	23.928	1.75	9.40	0.332

(a) Khan, 1982.

protein by the method of least squares. The linear curve shown in Figure 28 fits the equation

$$F(\nu) = 0.023\ M^{1/3} - 0.213 \qquad \ldots(11)$$

The values of $F(\nu)$ for the protein fractions A, B and C in Table X would correspond, according to equation 11, to 119, 94 and 67 kDa respectively. (see Table X). Further an empirical relationship between $F(\nu)$ and Stokes radius for the marker protein can be obtained by the method of least squares (see Figure 29). The linear curve fits the equation

$$F(\nu) = 0.192\ r\ (nm) + 0.049 \qquad \ldots (12)$$

The Stokes radii of the fractions A, B and C according to equation 12 would be 4.54, 4.08 and 3.48 nm respectively.

Corresponding to the Stokes radius of the main IgG binding protein fraction of 3.48 nm related hydrodynamic parameters were calculated.

The frictional ratio f/f_o of the IgG binding protein was calculated using the relation (Andrews,1970).

$$f/f_o = \frac{r}{(3\bar{\nu}_2\ M)\ 4\ \pi\ N)^{1/3}} \qquad \ldots (13)$$

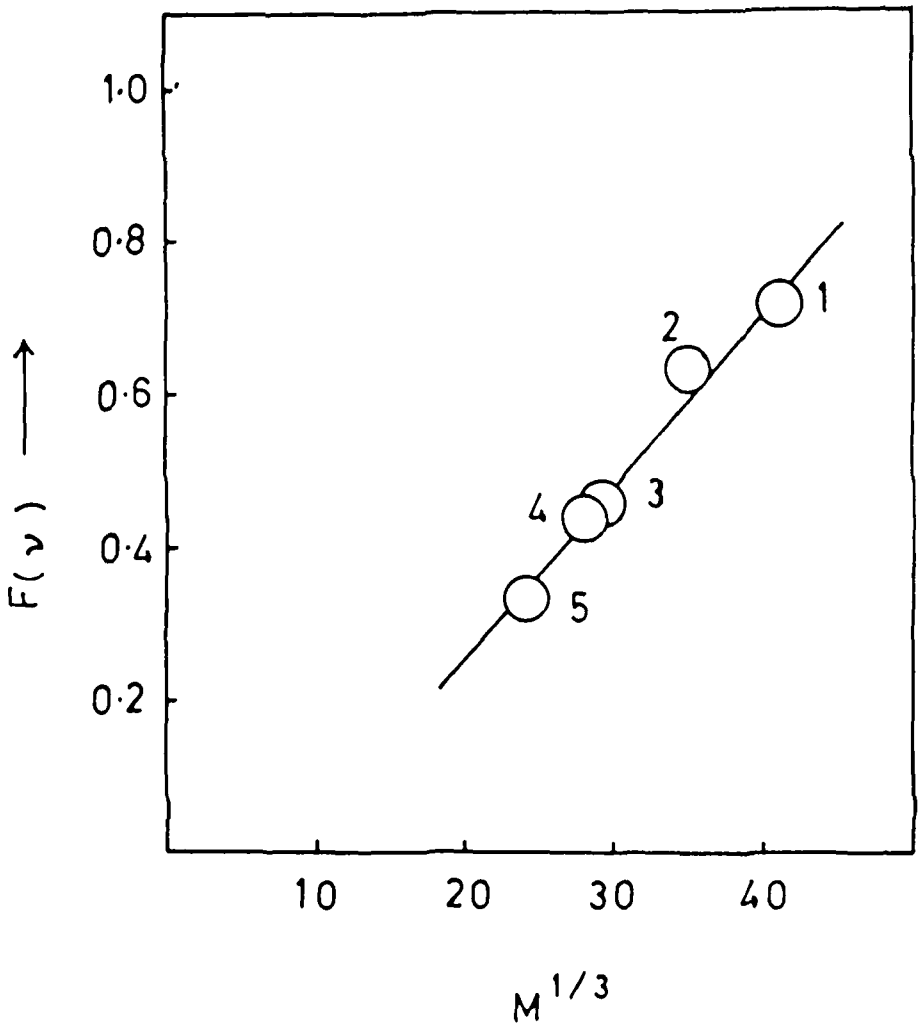


Figure 28.

Plot of F(ν) of marker proteins versus $M^{1/3}$.

The marker proteins (1) bovine serum albumin (2) ovalbumin (3) chymotrypinogen A (4) trypsinogen and (5) ribonuclease A were gel filtred on HPLC Shim Pack Diol 150 column equilibrated in 10 mM sodium phosphate buffer pH 7.5 containing 0.5 M NaCl. About 10 µg of marker proteins in 20 µl of buffer was applied on the HPLC column and the retention time of each protein determined. The method of calculation of F (ν) for marker proteins as well as membrane IgG binding protein is described in text.

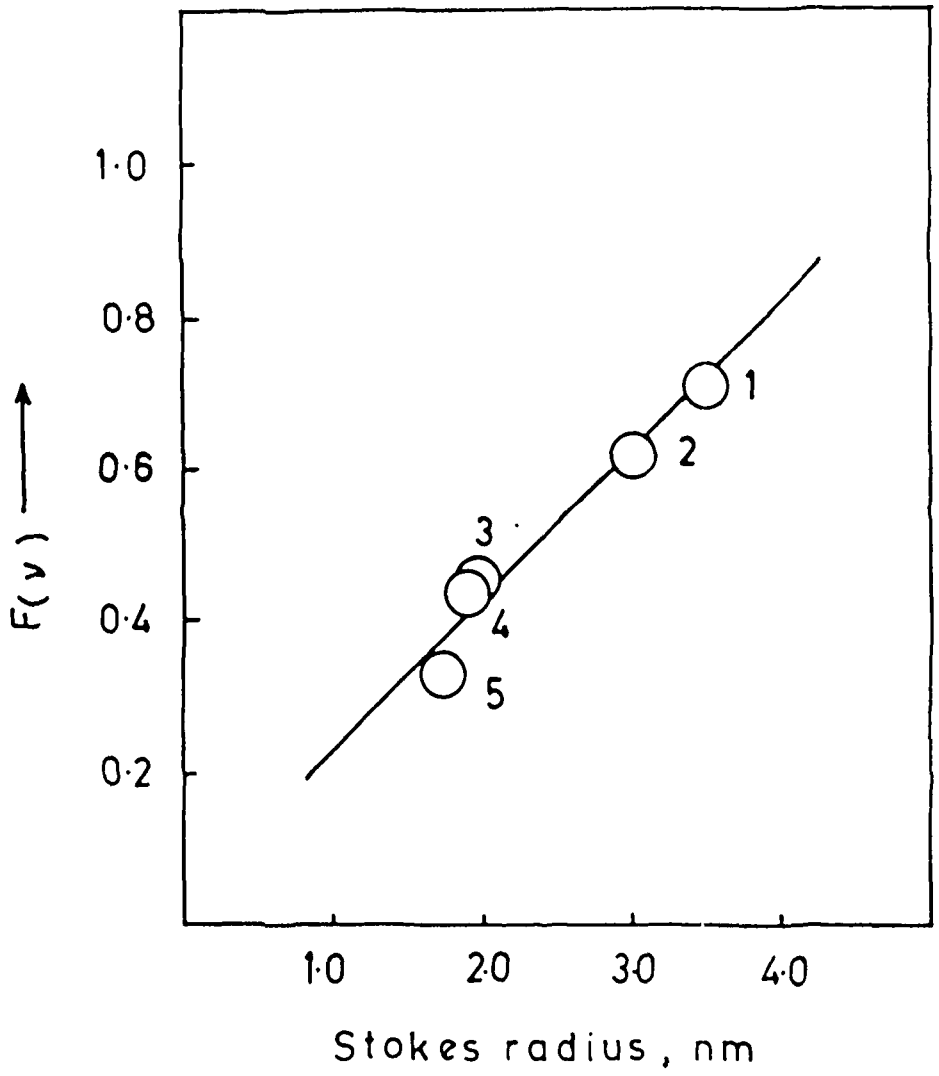


Figure 29.

Plot of F (ν) of marker proteins versus Stokes radius.

The marker proteins (1) bovine serum albumin (2) ovalbumin (3) chymotrypinogen A (4) trypsinogen and (5) ribonuclease A were gel filtred as described in the legend to Figure 28.

where N is a Avogadro's number (6.023×10^{23} per mole) and $\bar{v}_2$ is the partial specific volume of the IgG binding protein. As the partial specific volume of IgG binding protein is not known the partial specific volume (0.728) of human α_1 antitrypsin inhibitor was used in this calculation (Durchschlag, 1986). Human α_1 antitrypsin inhibitor also contains 11.5% carbohydrate which is similar to the presence of 11% carbohydrates on IgG binding protein. The molecular weight of the IgG binding protein was taken to be 67000 as determined by gel filtration. Using these values the frictional ratio of IgG binding protein was calculated with help of equation 12 as 1.3 .

The diffusion coefficient of the IgG binding protein was calculated by the help of following expression (Andrews 1970).

$$D = kT/6\pi\eta r \qquad \ldots (13)$$

where k is the Boltzman constant η is the coefficient of viscosity of the medium(i.e. 10 mM sodium phosphate buffer pH 7.5 containing 0.5 M NaCl) and T is the absolute temperature. With $K=1.386 \times 10^{-16}$ ergs/degree, η =0.012 poise, T=298 and the value of Stokes radius as 3.48×10^{-7} cm the value of diffusion coefficient for IgG binding protein was found to be 5.2×10^{7} cm^2/sec.

10. Association dissociation behaviour of lymphocyte membrane IgG binding protein:

The affinity purified IgG binding receptor was incubated in 0.5 N acetic acid, pH 2.8 for 16 hrs at 37°C and 20 µl of this solution was applied on Shim Pack Diol 150 column equilibrated with 10 mM sodium phosphate buffer pH 7.4 containing 0.5 M NaCl. The elution profile shown in Figure 30 was obtained. The major fraction (D) eluted with a retention time of 7.93±24 min constituted 86% of the total protein (see Table XII). The value of $F(\gamma)$ for fraction D was computed as described above and was 0.504 which according to equation 11 would yield a molecular wt. of 31000. The Stokes radii calculated from the value of $F(\gamma)$ according to equation 12 comes out to be 2.37 ± 0.17 min. The minor fraction (C) elutes with a retention time of 6.32 ± 0.19 min which would correspond to molecular weight of 72000 according to equation 11. The corresponding value of Stokes radius comes out to be 3.6 ± 0.17 nm. The molecular weight of protein fraction C is 2.3 times the molecular weight of fraction D. This would mean that the protein under fraction C may be the dimer of the protein fraction D. On reduction of temperature from 37°C to 4°C the elution profile of the acid treated IgG binding receptor is appreciably changed and from the readings of the integrated recorder the concentration of C, C' and D

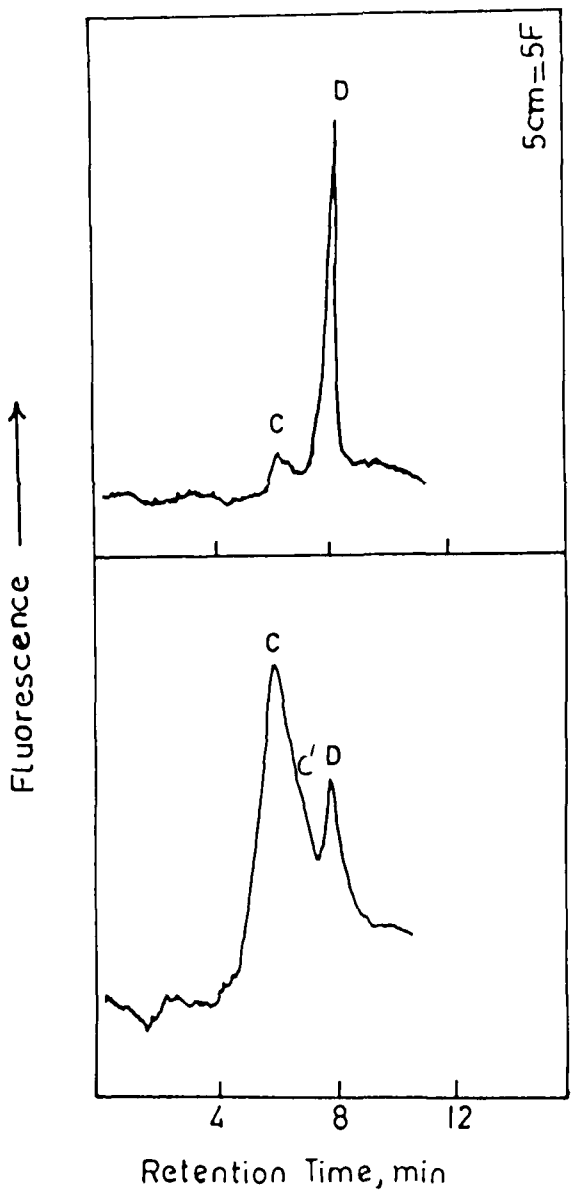


Figure 30

The effect of temperature on HPLC gel filtration of goat lymphocyte membrane IgG binding protein

The membrane IgG binding receptor in 0.5 M acetic acid was gel filtred on HPLC column equilibrated with 10 mM sodium phosphate buffer pH 7 4 containing 0.5 M Nacl. The protein was eluted with the same phosphate buffer at a flow rate of 1 ml/min. and detected spectrofluorometerically as described in the legend to Figure 27 The upper curve indicates the elution profile of 1 6 µg of membrane protein at 37°C whereas the elution profile given in the lower curve represents the chromatography of 2 1 µg of the lymphocyte membrane protein at 4°C

TABLE XII

Gel filtration of lymphocyte membrane IgG binding protein on HPLC Shim Pack Diol 150 column[a]

Peak	Retention time, min.	F(ν)	Relative conc.	Molecular wt.	Stokes Radius (nm.)
(a)					
C.	6.32±.19	0.740	14%	72000±7000	3.6±.17
D.	7.93±.24	0.504	86%	31000±4000	2.37±.17
(b)					
C.	6.28±.19	0.754	65%	75000 ±7000	3.68±.17
C'	7.00±.21	0.641	17%	52000±6000	3.09±.17
D.	7.99±.24	0.511	18%	32000±4006	2.41±.17

a, Details of peak shown in figure 30

comes out to be 65%, 17% and 18%, respectively. The retention times of the three fractions from the Shim Pack Diol column were found to be 6.28 ±0.19, 7.00 ±0.21, 7.99 ± 0.24 min which would yield $F(\gamma)$ values of 0.754, 0.641 and 0.511 respectively. From these gel filtration results the molecular weights as well as Stokes radius of C, C' and D were computed by the help of equation 11 and 12 respectively. The values of molecular weights of C, C' and D were 75, 52 and 32 kDa, respectively and the corresponding values of Stokes radii were 3.68, 3.09 and 2.41 nm. Thus the predominant form at 4°C is the protein fraction C. It should be noted that on reducing the temperature from 37°C to 4°C the protein fraction C increased from 14% to 65%. On the other hand the relative concentration of protein Fraction D with molecular weight of 31 kDa reduced from 86% to 18%. Further an additional hump (C') with a molecular weight of 52 kDa appeared at 4°C. These results do indicate association of 31 kDa species to a species of higher molecular weight upon decreasing the temperature from 37°C to 4°C.

The IgG binding protein eluting from affinity column by 0.5 N acetic acid was neutralized with 1M disodium dihydrogen orthophosphate and later dialysed against 2 mM phosphoric acid pH 2.8 containing 0.15 M NaCl and stored at three different temperatures for 15 hours. It was then analysed by gel filtration on HPLC Shim Pack Diol-150

column equilibrated in 2 mM phosphoric acid pH 2.8 containing 0.15 M NaCl. The results are shown in Figure 31. The main features of the elution profile as calculated by the CR3A integrator connected with the detector, are listed in Table XIII. As the column was equilibrated with acidic buffer equation.11,12 obtained with the marker proteins cannot be used in computing the molecular weights and Stokes radii from the retention times (or $F(\nu)$) given in Table XI.In fact the column characteristics were significantly altered by changing the buffer from 10 mM sodium phosphate pH 7.4 containing 0.5 M NaCl to 2 mM phosphoric acid pH 2.8 containing 0.15 M NaCl.

For example the retention time of ribonuclease A in sodium phosphate buffer, pH 7.4 was 9.40 whereas in the phosphoric acid pH 2.8 the value of retention time was 10.10 at 25°C. In general the average experimental error involved in the measurement of retention time of protein in the studies described in Table XIIIwas about 3%. The retention time of the protein fraction under peaks A is 5.52 min at 4°C. The retention times of B and C were 6.38 and 7.57 min ,respectively. The predominant species being protein fraction under peak C constituting 70% of the total IgG binding protein. On increasing the temperature from 4°C to 25°C the concentration of low molecular weight protein with retention time of about 7.75 min is

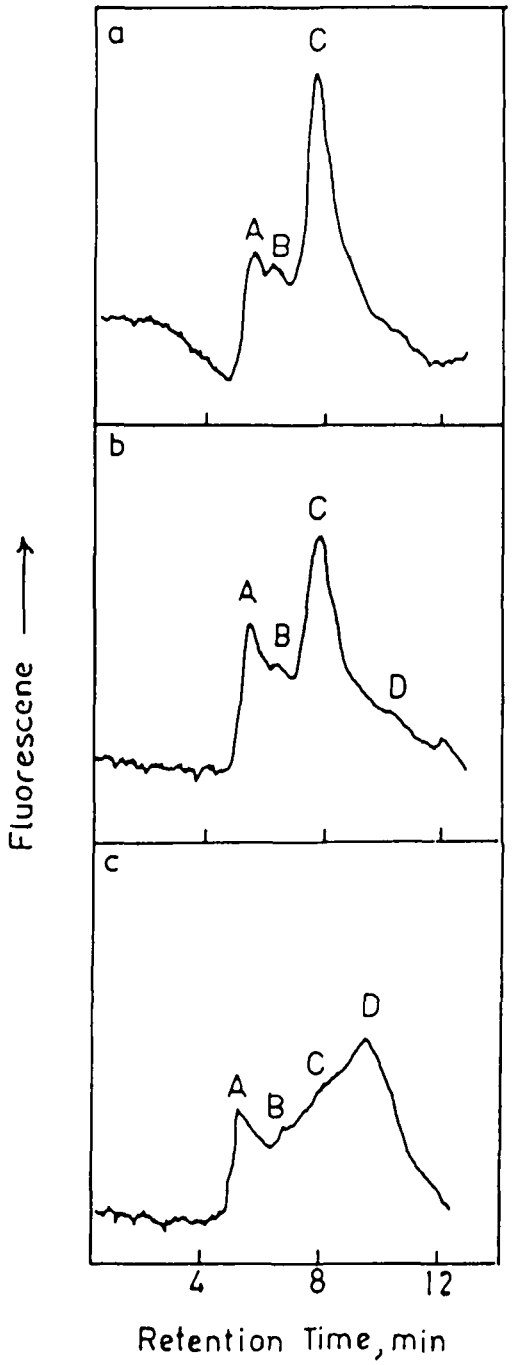


Figure 31

Effect of temperature on the gel chromatography of the membrane IgG binding protein from lymphocytes

About 3 7 µg of IgG biding protein in 2 mM phosphoric acid pH 2 8 containing 0 15 M NaCl was gel filtred on HPLC Shim Pack Diol 150 column (79 x 25 cm) equilibrated in the same solvent operating at a flow rate of 1 ml/min The protein peak were detected spectrofluorometerically at 354 nm Keeping excitation at 280 nm The slit used for both the beams was 15 nm The peaks were recorded automatically by C-R3A integrator (a) at 4°C (b) at 25°C (c) at 37°C The ordinate scale represents 5 cm=5F

TABLE XIII

Effect of temperature on gel filtration of lymphocyte membrane IgG binding protein on Shim Pack Diol 150 column. [a]

S.No.	Condition	Peak	Retention Time, min.	Relative Conc.
1.	pH 2.8	A	5.52±.17	16
	Temp 4°C	B	6.38±.19	14
		C	7.57±.23	70
2.	pH 2.8	A	5.23±.16	22
	Temp 25°C	B	6.34±.19	13
		C	7.75±.23	56
		D	10.06±.30	9
3.	pH 2.8	A	5.37±.16	17
	Temp 37°C	B	6.32±.19	12
		C	7.42±.22	12
		D	9.36±.25	58

a. Details of peaks shown in Figure 31.

substantially reduced (from 70% to 56%) but the relative concentration of high molecular weight aggregates (A) increased from 16% to 22%. A low molecular wt. species with a retention time of about 10.0 min appeared at high temperature. The concentration of fraction B remains unaltered by increasing temperature. Further, increase in temperature from 25°C to 37°C caused substantial increase in the concentration of the low molecular weight species D: the relative concentration increased from 9% to 58%. Interestingly, the relative concentration of the fraction C which was predominant at 25°C decreased from 56% to 12% on raising the temperature from 25°C to 37°C. These results suggest temperature dependence of IgG binding protein. At pH 2.8 increase in temperature from 4°C to 37°C produced marked disaggregation of the IgG protein.

IV . DISCUSSION

The results presented in this thesis provided definite evidence for the presence of IgG binding protein on the surface of goat peripheral blood lymphocytes. It should be pointed out that homologous agg IgG was used in these studies because the extent of its binding to cells is expected to be substantially higher. The binding of heat agg IgG to cells was saturable which is consistent with the specific nature of the binding of the Ig by cell surface receptors. The amount of agg IgG required to saturate the binding site on cells was 0.7 μg /ml which is equivalent to about 5 x 10^7 moles of IgG monomer (See Figure 14). This would yield 10^7 moles of receptor per cell which is three to four order of magnitude higher than that found for human leukemia receptor containing 10^4-10^5 receptor/cell(Segal et al., 1979: Alexander et al. , 1979) . We have assumed that each IgG monomer in agg IgG binds to the cell surface receptor under saturating conditions which may not be true. Since an oligomer may be attached to a cell surface by only one or two IgG monomers the rest of the IgG monomers may be redundant. Thus the total number of the receptor per cell on goat peripheral blood lymphocytes is likely to be substantially lower.

The binding of heat agg IgG and its derivatives to cell surface receptors as studied by ELISA was found to be

sensitive to pH and ionic strength. By the use of heterologous agg IgG instead of homologous goat IgG the error due to binding of anti IgG to surface Ig was avoided. That the heterologous agg IgG also bound to goat lymphocytes shows that the IgG binding receptor recognised IgG regardless of its species.

At a given pH the IgG binding to cell surface receptor was maximum for heat agg IgG followed by IgG monomer and the binding was small but significant in case of the $F(ab_2')$ fragment of human IgG (See Figure 18). Another notable observation was that the binding of heat agg IgG to cell surface receptor was more sensitive to pH than the binding of IgG monomer. Maximum binding of the agg IgG occurred at pH 6 in the pH range 3-8. Interestingly while goat peripheral blood lymphocyte receptor retains substantial binding activity even at pH 8, the intestinal brush border membrane receptor from neonatal rat was inactivated at pH 7.4 (Wallace and Rees, 1980). Further chick yolk sac receptor is inactivated at pH 8.0 (Tressler and Roth 1987). Acid treatment at pH 3.0 does not abolish binding activity of goat peripheral blood lymphocyte receptor at which pH macrophage IgG receptor is completely inactivated (Mellman and Unkeless 1980). One of the striking observation is that the truncated murine IgG receptor lacking the cytoplasmic and

transmembrane domains shows optimal binding at pH 3 which decreases rather rapidly on increase in pH to pH 10. (Qu et. al., 1988). The receptor in absence of detergent may exist as a protein micelle due to aggregation via interdomain interactions involving hydrophobic transmembrane domains. These micelles would dissociate at low pH values resulting in loss of cooperative binding. The latter has been found, to be necessary for interaction of the receptor with the immune complex. The dependence of binding of monomeric IgG to goat peripheral blood lymphocyte receptor on pH was found to be small. As pointed out above the binding of $F(ab_2')$ to the receptor is small and is completely abolished at extreme pH values. The observed pH dependence of the binding of IgG monomer and its derivatives to cell surface receptor (See Fig.18) can be attributed to the pH induced structural changes in receptor and or IgG and its modified form. IgG monomer and $F(ab_2')$ differ only in Fc region so that the difference in pH dependence of their binding to lymphocytes are likely to be attributable to different cell surface receptors or to different domains of the same receptor. Interestingly separate receptor for $F(ab_2')$ and Fc region have been recognised on human platelets (Vancura and Steiner, 1987). Even on one cell two different classes of IgG FcR have been detected (Jones et al., 1985). Different domains of the same receptor may also be binding to IgG and $F(ab_2')$

both as they have similar domain structure. $F(ab_2')$ fragments of polyclonal human IgG, and human IgG myeloma have the ability to inhibit the binding of protein A to immobilized human Fc fragments, suggesting that these immunoglobulins express, within their variable regions, determinants that possess Fc γ like properties (Biguzzi, 1982).

The binding of IgG monomer by GPBL was reduced only by 50% in the presence of protein A which is known to interact with the C_H2 and C_H3 domains of the molecule. (Biguzzi, 1982). This shows that all the receptor are not bound to the IgG monomer through the Fc region. Some of them are bound to IgG in the region different from the protein A binding site of the Fc of IgG. The cell surface receptor binding $F(ab_2')$ is unlikely to recognize Fc portion of the IgG molecule but will interact through its $F(ab_2')$ portion. It is possible that pH dependence of the binding of IgG through $F(ab_2')$ receptor is counter balanced by the pH dependence of its binding through the Fc receptor.

The binding of aggregated IgG to GPBL remained almost unaffected by protein A, presumably because of the inaccessibility of the latter to the C_H2-C_H3 domains in aggregated IgG. The marked pH effect on the binding of aggregated IgG (Fig. 18) may be ascribed to the pH induced

structural changes in aggregated IgG, resulting in increased accessibility of binding site on IgG molecules for cell surface receptor. These changes appear to be cooperative and may involve the dissociation of a "acidic" and "basic" group with apparent pK of 3.6 and 7.4 respectively.

The binding of IgG to cell surface receptor was also found to depend on ionic strength, decreasing gradually upon increasing the concentration of NaCl from 0-0.8 M. The binding of aggregated IgG was significantly higher than the binding of monomeric IgG near physiological ionic strength. For rat intestine receptor the degree of binding was independent of the buffer ion used (eg. Tris/HCl, phosphate or NaCl in combination of either of these)(Wallace and Rees 1980).However maximum binding occured at a relatively low ionic strength with an optimum binding at 60 mM Tris/HCl buffer (Wallace and Rees, 1980). At low ionic strength the binding of murine aggregated IgG_{2b} to its receptor on human monocyte cell lines U937 is enhanced whereas the binding of monomeric IgG_{2a} to its receptor is not affected (Jones et al., 1985). Restricted chemical modification of acidic side chains of Fc gamma fragment abolishes its binding to human neutrophil Fc receptor (Bradgo et al.,1982). Polycations such as polylysine as well as polyanions like dextran sulphate inhibit the binding of immune complexes to FcR. (Fornusek

and Vetvicka, 1984). All these facts suggest that there exists important ionic interactions between IgG and its receptor. Whether the pH or ionic strength dependence of the binding of IgG to the receptor is due to the effect on receptor or on the IgG molecule remains to be investigated. In conclusion, our results suggest that the binding of IgG monomer, IgG aggregated and $F(ab_2')$ to cell surface receptor is polar in nature.

Goat peripheral blood lymphocyte membrane IgG binding protein was prepared both from cell homogenate as well as isolated membrane by repetitive affinity chromatography on agg IgG Sepharose 4B column. Both the receptor preparations were identical in (a) reactivity towards agg IgG and con-A Sepharose gel (b) subunit molecular weight (c) in gel filtration behaviour and (d) finally in their tendencies to undergo pH and temperature dependent aggregation.For the isolation of IgG binding protein, lymphocytes or its isolated membrane was solubilized in 10 mM sodium phosphate buffer containing 0.15 M NaCl, 2 mM PMSF, 3 mM EDTA, 10 mM iodacetamide and 0.5% NP-40. NP-40 at this concentration does not solubilize nuclear membrane thus protecting the release of nucleic acids into the cell homogenate (Takacs, 1980,). At this detergent concentration NP-40 effectively solubilized membrane protein without effecting their ability to bind specific ligands.

The yield of IgG binding protein from whole cells as well as its isolated membrane was less than 1% which is similar to that found by Takacs (1980) for human peripheral blood lymphocyte receptor. Similar yield was found from human peripheral mononuclear cells (Sandor, 1978). Vojtiskova and Franeck (1984) isolated crude receptor by affinity chromatography from the protein fraction shed by pig lymphocytes following a temperature shift : the protein yield was around 1%. However the yield of receptor from guinea pig peritoneal macrophage was substantially low, 0.1% (Janusz et al., 1986). When monoclonal antibodies against receptor was used for immuno precipitation of the receptor the yield of mouse macrophage receptor was 0.01%(Mellman and Unkeless,1980).

The IgG binding receptor isolated from the goat peripheral blood lymphocytes was found to be a glycoprotein devoid of sialic acid residues. The hexose content of the receptor was found to be 11% which is lower than that (30-34%) found for human placental (Mikulska et al., 1985) and murine lymphocyte receptors (Zikan et al., 1986). The nature of the carbohydrate moeities in goat peripheral blood lymphocyte receptor was determined by chromatography on Con-A-Sepharose 4B. The receptor specifically interacted with the affinity column suggesting the presence of glucose/mannose residues since

Con-A is known to interact specifically with these residues (Leiner et al., 1986).

Results on light absorption and fluorescence measurement showed that IgG binding protein of goat peripheral blood lymphocyte contained among others tryptophan residues which have been recognized in the receptors from mouse macrophage cell line (P388 D_1, (Lewis et al, 1986) J774 cell line, human T cell line S49.1, (Ravtech et al., 1986) and NK cells (Simmons and Seed, 1988).

As judged by the SDS-PAGE the subunit molecular weight of the receptor was found to be 14 kDa which is similar to Mr (13 kDa) of chicken erythrocyte receptor (Manghi et al, 1987) and rat thymocyte receptor (12 kDa) (Bezvershenko et al, 1980).

The IgG binding receptor being a membrane protein expectedly showed pronounced tendency of aggregation in aqueous solution which was found to depend among others on pH and temperature. In 10 mM sodium phosphate pH 7.4 alone receptor solution was turbid and the turbidity decreased upon increasing NaCl concentration.

When IgG binding protein solution eluted with 0.5 M acetic acid pH 2.8 from the affinity column was

neutralized to pH 7.4 at 25°C and the solution analysed by HPLC gel filtration three fractions A, B and C with molecular weights of 67, 94 and 119 kDa were obtained; 67 kDa species formed the major (60%) fraction. Upon exposure of the Ig receptor to pH 2.8 and 37°C the receptor eluted in two fractions D and C with molecular weights of 31 and 67 kDa; the fraction A and B were absent. These results showed that acid and heat treatment caused dissociation of the receptor aggregates with molecular weight of 119 (A) and 94(B) kDa. These results taken together with the fact that subunit molecular weight of the receptor was determined by SDS-PAGE to be 14 kDa suggested that the IgG binding protein from goat peripheral blood lymphocytes exhibit pH and temperature dependent aggregation so that A, B and C are respectively tetramer, trimer and dimer of D which with molecular weight of 31 kDa is a dimer of 14 kDa species. The observation that the subunit molecular weight by SDS-PAGE remains 14 kDa with and without 2- mercaptoethanol showed that the two subunit in the dimer D are held together only by non-covalent forces.

The 67 kDa species which is the dominant form of the IgG binding protein at 25°C and at pH 7.4 was eluted from the gel filtration column with a Stokes radius of 3.48 nm which would correspond to a diffusion coefficient of

$5.2x10^{-7}$ cm^2/sec and a frictional ratio of 1.3 . These hydrodynamic parameters are consistent with a nonglobular conformation of the IgG receptor at pH 7.4.

Upon neutralization of the low pH solution of the IgG binding receptor to pH 7.4 and subsequent acidification to pH 2.8 at three different temperatures namely 4^oC, 25^oC and 37^oC the IgG binding receptor was found to contain four molecular species A, B, C, D of which fraction C was dominant at low temperature i.e. 4^oC and 25^oC. Interestingly the effect of temperature on the concentration of fraction A and B was small so that the two fractions were almost invariant. It therefore seems that the temperature had effect only on relative concentration of C and D, the formation of the fraction with highest retention time and hence lower molecular weight was favoured by rise in temperature to 37^oC. These results also suggests the temperature dependence of the association of the acid treated IgG binding receptor. Since increase in temperature favours dissociation the process seems to be endothermic.

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BIOGRAPHY

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LIST OF PUBLICATIONS/PRESENTATIONS

PUBLICATIONS

1. "Binding of Immunoglobulin G to peripheral blood lymphocytes." K. Hajela and A. Salahuddin. (1988) Immunol Lett, 19. 159-163.

2. "Isolation and characterization of IgG binding protein from lymphocytes". K. Hajela and A. Salahuddin. Communicated to Biochem. Biophys. Res. Commun. in May, 1989.

PRESENTATIONS:

1. "Studies on IgG binding protein from lymphocyte membrane." Krishnan Hajela and A. Salahuddin. Oral Presentation at the 57th annual meeting of S.B.C. (I), held at Department of Biochemistry V.P. Chest Institute University of Delhi. On October 9-12, 1988.

2. "Membrane protease activity in peripheral blood lymphocytes." Mrs. Zoya Galzie and Krishnan Hajela. Presented at the 57th Annual Meeting of the S.B.C. (I) held at the Department of Biochemistry V.P. Chest Institute, University of Delhi. On October 9-12,1988.

3. "Interaction of FcR with Immunoglubulin and its derivatives." Krishnan Hajela. Accepted for presentation at the 56th Annual Meeting of the S.B.C. (I) held at Department of Chemistry. Sir Venketeshwara University, Tirupati on December 29-31,1987.

4. "Association and dissociation of IgG binding protein from goat lymphocytes." Krishnan Hajela and A. Salahuddin presented at the 56th Annual Meeting of S.B.C. (I) held at Department of Biochemistry Sri Venkateshwara University, Tirupati. On December 29-31, 1987.

5. "Isolation and partial characterization of Fc Receptors from peripheral blood lymphocytes." Krishnan Hajela and A. Salahuddin. presented at 55th Annual Meeting of S.B.C. (I) held at Department of Biochemistry, Kerala University, Trivendrum on December 15-17, 1986.

6. "Use of heat aggregated Immunoglobulin G-Sepharose column for the isolation of Fcγ rabbit lymphocytes." Krishnan Hajela and Naushin Haleem Khan. Abstract No.430. Presented at 54th Annual Meeting of the S.B.C. (I) held at the Department of Biochemistry G.B. Pant University of Agriculture and Technology, Pantnagar on November 2-9, 1985.

7. "Controlled digestion of human IgG by papain." Miss Naushin Haleem Khan and Krishnan Hajela. Abstract No.111. Presented at 53rd Annual Meeting of the S.B.C. (I) Held at the Department of Biochemistry. V.P. Chest Institute, University of Delhi. On October 12-14, 1984.

Immunology Letters, 19 (1988) 159–162
Elsevier

IML 01126

Binding of immunoglobulin G to peripheral blood lymphocytes

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(Received 19 August 1988; accepted 26 August 1988)

1. Summary

The specific and saturable binding of FITC conjugate of aggregated goat IgG to goat peripheral blood lymphocytes was studied in PBS containing 1% BSA. The polar nature of the specific interaction of heterologous aggregated IgG, IgG monomer and its fragment F(ab$_2'$) with the cells was studied by ELISA using the peroxidase conjugated F(ab$_2'$) of anti-human IgG under different conditions of pH and ionic strength.

2. Introduction

Immunoglobulins may specifically interact with lymphocytes either through its Fc portion or antibody combining site in the Fab region of the molecule. The binding of IgG through Fc portion to the specific cell surface receptor is implicated in such processes as antibody dependent cell mediated cytotoxicity (ADCC), suppression of antibody synthesis in B cells and phagocytosis in macrophages [1, 2]. In ADCC the receptor appears to bind C_H2 and C_H3 domains of the target IgG [3]. Although the IgG-receptor interaction is known to depend on pH [4–6] and ionic strength [4], observations made on different preparations of the receptor are often conflicting. Thus while the receptor from intestinal brush border membrane of neonatal rats was optimally active at pH 6.6 and inactive at pH 7.4 [4], that from mouse macrophage continued to show substantial activity up to pH 9.0. The receptor activity was completely abolished at pH 10.0 [5]. The loss of receptor activity that occurred by exposure to alkaline pH was, however, reversible [6]. In view of the suspected species-dependent pH stability of the receptor and the suggested polar nature of IgG-receptor interaction [4] we have studied the binding of IgG and its derivatives with the membrane bound receptor on the intact goat peripheral blood lymphocytes under various conditions of pH and ionic strength using ELISA as employed earlier [7, 8].

Key words: IgG binding; pH effect; Goat lymphocytes; ELISA

Abbreviations: FcR, receptor for Fc portion of immunoglobulin; ADCC, antibody dependent cellular cytotoxicity; ELISA, enzyme linked immunosorbent assay; Conj-(Fab$_2'$), peroxidase conjugated F(ab$_2'$) fragment of anti-human IgG; NP-40, noniodet P-40; FITC, fluorescein isothiocyanate; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis; GPBL, goat peripheral blood lymphocytes; PBS, phosphate-buffered saline, pH 7.4; DEAE, diethyl amino ethyl; OPD, orthophenylene diamine.

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3. Materials and Methods

3.1. *Materials*

Preparations of protein A, peroxidase conjugated F(ab$_2'$) fragment of antihuman IgG (Conj-Fab$_2'$), pepsin, Histopaque-1077, orthophenylene diamine (OPD), noniodet P-40 (NP-40), fluorescein isothiocyanate (FITC), thimerosal, DEAE-cellulose, and Sephadex G-200 (Sigma) were used.

3.2. *Isolation of IgG and preparation of its derivatives*

Human IgG isolated as described earlier [9] gave, as expected, two protein bands in SDS-PAGE. Aggregated IgG was prepared by heating 1% protein so-

lution in 10 mM sodium phosphate buffer, pH 7.4, at 60–65 °C [10] and subsequent fractionation on Sephadex G-200 column (2.4×88 cm). $F(ab'_2)$ fragment of IgG was prepared by controlled peptic digestion [11].

3.3. *Enzyme-linked immunosorbent assay*

Goat peripheral blood lymphocytes (GPBL) were isolated by Histopaque-1077 density gradient centrifugation [12] and suspended in PBS, pH 7.4, containing 0.2% sodium azide which inhibits endocytosis [13]. In order to determine binding of heterologous IgG (human) to GPBL according to Kurita et al. [7], cells were fixed in ELISA plate wells with 0.5% glutaraldehyde [14], washed with PBS containing 0.1% NP-40, treated with BSA (10 mg/ml), to prevent nonspecific binding, washed with PBS, treated with IgG (0.01 mg) for 2 h and again washed with PBS. The heterologous IgG instead of goat IgG was taken to exclude the possibility of interaction with membrane bound surface IgG on mature lymphocytes. Conj-$F(ab'_2)$ solution was diluted 400 fold with PBS containing 1% BSA, 0.01% thimerosal and the solution added to the wells and incubated for 2 h. After washing with PBS containing 0.1% NP-40, 0.2 ml of substrate solution (0.05% H_2O_2 in 0.2 M sodium citrate, pH 5.0 and OPD 0.1%) was added. After incubation at 37 °C for 5 min reaction was stopped with 3N HCl and the colour intensity measured at 492 nm. Absorbance at 492 nm was a measure of IgG binding to lymphocytes. BSA was included in the assay mixture to block nonspecific binding sites on cell surface. In control wells IgG or its derivatives were omitted.

3.4. *Aggregated IgG binding assay*

Saturation binding curve was obtained with aggregated IgG instead of monomeric IgG since the former interacts with the cells with higher avidity than the latter [15]. Goat IgG was conjugated with FITC in 0.2 M sodium carbonate buffer, pH 9.0, for 30 min at 37 °C [10] and resulting FITC-IgG solution separated from unreacted FITC by gel filtration on Sephadex G-25 column (2×20 cm). The conjugated IgG was further fractionated on DEAE cellulose column (2.2×15 cm), equilibrated with 0.01 M sodium phosphate buffer, pH 8.0. The bound protein was eluted with different concentrations of sodium chloride and the fractions obtained by elution with 0.5 M NaCl were pooled and heat-aggregated as described above. In order to determine the binding of FITC-IgG (agg) to the GPBL, 4.5×10^7 cells were treated with increasing concentrations of FITC-IgG-(agg) at 4 °C for 2 h in buffer A (PBS, pH 7.4, containing 1% BSA and 0.2% NaN_3), then washed with buffer A twice and then pelleted by centrifugation at 2000 rpm for 10 min and the bound FITC-IgG (agg) was dissociated with 0.5% NP-40. The suspension was centrifuged at 2000 rpm for 10 min and FITC-IgG-(agg) in the supernatant measured spectrofluorometrically at 520 nm with an excitation wavelength of 492 nm. In another set the conjugated protein was taken with 100-fold excess of unconjugated aggregated IgG and incubated with lymphocytes at 8 °C in buffer A. After washing with buffer A the amount of FITC-IgG-(agg) bound to cells in the presence of 100-fold excess of unconjugated IgG was estimated spectrofluorometrically as described above after dissociating in 0.5% NP-40.

4. Results and discussion

4.1. *Binding of aggregated IgG to lymphocyte receptors*

That aggregated IgG was specifically bound to cell surface receptor can be seen in Fig. 1, depicting plot of total IgG versus bound IgG. The binding of FITC-IgG agg to GPBL (4.5×10^7) in PBS increased considerably with the increase in the concentration of aggregated IgG, as can be seen in Fig. 1. The curve slopes off at 4.3 nm of the aggregated protein, suggesting saturation of specific binding site on the cells with IgG. The results suggest that about 10^7 moles of ligand are bound to one cell.

Binding of human IgG monomer, aggregated IgG and $F(ab'_2)$ fragment to peripheral goat blood lymphocytes was studied by ELISA using peroxidase conjugated $F(ab'_2)$ of antihuman IgG under different experimental conditions of pH and ionic strength. The binding of $F(ab'_2)$ fragment was relatively smaller but experimentally significant.

4.2. *Effect of pH*

The binding of IgG and its derivatives to the lym-

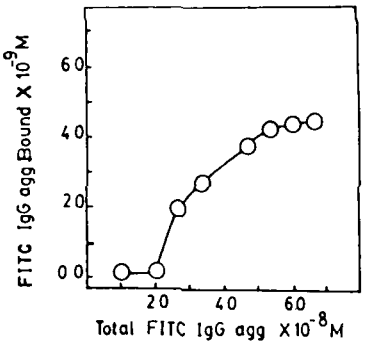

Fig. 1. Binding of FITC conjugate of aggregated IgG to goat lymphocytes. Increasing concentration of conjugated protein was incubated with 4.5×10^7 GPBL in PBS, pH 7.4, containing 1% BSA and 0.2% NaN_3 at 8 °C for 2 h and the bound protein determined spectrofluoremetrically at 520 nm (excitation 492 nm) as described in the text. Each observation represents the average of two independent experiments

phocytes was studied at different pH values in the range of 3–8. Sodium phosphate buffer (0.06 M) was used for maintaining pH 6, 7 and 8. Below pH 6.0 sodium acetate (0.06 M) buffer was employed. IgG or its derivatives were incubated with fixed cells at the desired pH for 2 h at 30 °C and the binding measured by ELISA. The results are graphically shown in Fig. 2. Here it should be pointed out

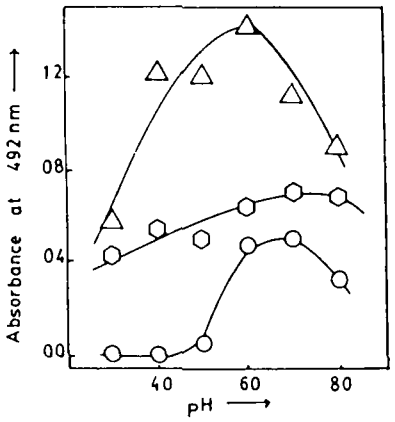

Fig. 2. Effect of pH on binding of immunoglobulin and its derivatives to lymphocyte receptors. Ten μg of aggregated IgG (▵) monomeric IgG (○) and its F(ab′)2 fragment (○) were incubated *with 10^5 cells in 60 mM sodium phosphate buffer (pH 6–8) or* 60 mM sodium acetate buffer (pH 3–5) for 2 h and the bound protein measured by ELISA using peroxidase conjugated F(ab′)2 of antihuman IgG as described in the text. Each observation represents the average of three independent experiments

that after exposure of cell-IgG complex to the desired pH for 2 h it was allowed to react with Conj-F(ab′)2 in PBS (pH 7.4) for 2 h. Some reversible changes in binding might have occurred. It can be seen in Fig. 2. that maximum binding occurred with aggregated IgG followed by IgG monomer and F(ab′)2. Furthermore, the pH dependence of aggregated IgG is more pronounced than the binding of IgG monomer to cells in the entire pH range (i.e., 3–8). Maximum binding took place at pH 6.0, below or above which the changes in pH cause considerable reduction in binding. Strikingly, the receptor activity is not abolished even at pH 3.0. In contrast, the macrophage receptor activity was lost at pH 4.0 [5]. Unlike aggregated IgG the binding of monomeric form to cells is less sensitive to pH. The binding decreased gradually and monotonically as the pH decreases from 8 to 3. The pH dependence of interaction of F(ab′)2 with lymphocytes is significant. The interaction is completely abolished at extreme acid pH values and maximum binding occurred at near neutral pH. The observed pH dependence of the binding of IgG monomer and its derivatives to cells' surface receptors (Fig. 2) can be attributed to the pH induced structural changes in receptor and or IgG and its modified forms. *IgG monomer and F(ab′)2 differ* only in Fc region so that the differences in pH dependence of their binding to lymphocytes are likely to be attributable to different cell surface receptors or to different domains of the same receptor. That Fc region alone is not involved in the binding of IgG monomer by GPBL is shown by our observation that IgG binding was reduced only by 50% in the presence of protein A, which is known to interact with the C_H2 and C_H3 domains of the molecule [19]. The cell surface receptor binding F(ab′)2 is unlikely to recognise Fc portion of the IgG molecule but will interact through its F(ab′)2 portion. It is possible that pH dependence of the binding of IgG through F(ab′)2 receptor is counter-balanced by the pH dependence of its binding through the Fc receptor. Interestingly, separate receptors for F(ab′)2 and Fc regions have been recognised on human platelets [17]. Even on one cell line two different classes of IgG FcR have been detected [18].

The binding of aggregated IgG to GPBL remained almost unaffected by protein A, presumably because of the inaccessibility of the latter to the C_H2–C_H3 domains in aggregated IgG. The marked pH effect

in the binding of aggregated IgG (Fig. 2) may be ascribed to the pH-induced structural changes in aggregated IgG, resulting in increased accessibility of binding sites on IgG molecule for cell surface receptor. These changes appear to be cooperative and may involve the dissociation of a "acidic" and "basic" group with apparent pH of 3.6 and 7.4, respectively. The GPBL receptor appears to be different from brush border membrane receptor macrophage receptor and chick yolk sac receptor. The brush border receptor was inactivated at pH 7.4 [4] whereas the GPBL receptor for monomer retains all its activity. The goat lymphocyte receptor retains substantial activity at pH 3.0, whereas the macrophage receptor was completely inactivated [5]. Also at pH 8.0 the chick yolk sac receptor is inactivated [16] but the GPBL receptor remains active.

4.3. *Effect of ionic strength*

The effect of ionic strength on the binding of immunoglobulins and its derivatives, namely aggregated IgG and F(ab_2), was investigated in 10 mM sodium phosphate buffer, pH 7.0, containing different concentrations of NaCl (0-0.8 M). The results are shown in Fig. 3. Here again binding was found to depend on ionic strength, decreasing gradually upon increasing the concentration of NaCl from 0-0.8 M. The ionic strength of the buffer is 0.02. Below ionic strength of 0.2 the binding decreased on increasing ionic strength. In spite of scatter in data obtained for binding of aggregated IgG this conclusion remains unaltered. The binding of aggregated IgG was significantly higher than that of monomeric IgG near physiological ionic strength.

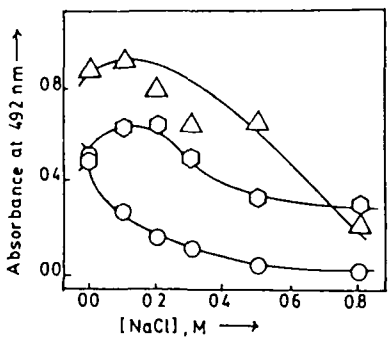

Fig 3 Effect of ionic strength on binding of immunoglobulin and its derivatives to lymphocyte receptors Ten μg of aggregated IgG (Δ), monomeric IgG ($\bigcirc$) and its F(ab_2) fragment (o) were incubated with 10 cells in 10 mM sodium phosphate buffer containing 0 0-0 8 M NaCl for 2 h and the bound protein measured by ELISA using peroxidase conjugated F(ab_2) of antihuman IgG as described in the text Each observation represents the average of three independent experiments

In conclusion, our results suggest that the binding of IgG monomer, IgG aggregate and F(ab_2) to cell surface receptor on GPBL is polar in nature and that there may be more than one receptor on GPBL.

Acknowledgements

Thanks are due to DST for financial assistance and to CSIRO for fellowship (K.H.) and to Aligarh Muslim University, Aligarh for facilities.

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